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ABSORPTION OF ENZYMES AND OTHER PROTEINS BY BARLEY ROOTS[1, 2]

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As part of a program of investigation on the characteristics of enzyme reactions at surfaces (9, 16, 17) it occurred to us to examine the pH-optimum for enzyme activity of enzymes adsorbed on the negatively charged (6) surface of plant roots. It was found at once, however, that the amount of lysozyme absorbed by barley roots was as high as one per cent of the dry weight of the roots, and we next sought to determine if the absorbed macromolecules could be detected in cross sections of roots which had been exposed, either excised or whole, to solutions of these proteins. In order to do so the proteins were first labeled with C^{14} carbon, and radioautographs of thin sections of roots previously exposed to solutions of labeled protein were obtained. Having established that C^{14} was found in roots so treated, the rates of protein uptake by roots were measured and the amounts compared with the exchange capacity of the roots. Any attempt, therefore, to interpret kinetic data dealing with the absorption of enzymes must take into consideration that the absorbed enzymes are distributed in, as well as on, the root.

Incidentally, although the uptake of substances by plants is generally taken as being confined to low molecular weight substances such as inorganic ions and sugars, it has been known for nearly 30 years that both exceedingly large molecules such as ovalbumin, and viruses can be taken up by some plants and translocated to aerial portions (20).

MATERIALS AND METHODS

Most of the uptake experiments were conducted with excised barley roots, grown and prepared as described elsewhere (12). The freshly excised, washed roots, 2 g, were placed in 25 ml screw-cap polyethylene bottles with dilute buffer solutions, 10 ml, containing crystalline lysozyme or human hemoglobin, and the partially filled bottles were repeatedly inverted slowly for adequate mixing by being mounted on a moving wheel (14). After various times of mixing at 24° ± 1° C, the roots were drained free of liquid by gravity and the liquid was analyzed for non-absorbed protein by means of a spectrophotometer (14). In the case of whole plants the roots were dipped in protein solution in plastic tubes and the roots were agitated gently by mildly shaking the tube.

Proteins labeled with C^{14} radioactive carbon were prepared by Dr. H. Tarver from 2-C^{14} labeled glycine anhydride by a procedure described by Margen and Tarver (19). The glycine contained 100 μc in 8.6 mg and the amount of activity added as the anhydride to 39.5 mg hemoglobin and 43.5 mg lysozyme was 8.4 μc to each. The reacted proteins were dialysed against M/100 glycine to displace any absorbed, unreacted labeled glycine and then against water (lysozyme) or M/1,000 sodium bicarbonate (hemoglobin). The protein solutions were quickly frozen with the aid of dry ice and stored frozen. The solutions were again dialyzed immediately prior to use. Analysis showed an activity of 0.04 μc/mg hemoglobin and 0.08 μc/mg lysozyme or in other words the labeling reaction was 20 per cent and 40 per cent efficient, respectively. Lysozyme and ribonuclease were randomly labeled with tritium by exposure to tritium gas by the New England Nuclear Corp., Boston (cf 21). These proteins were freed from any low molecular weight

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material immediately before they were used in an experiment by dialysis for 48 hours in cellophane tubing.

Barley roots, after being placed in C^{14} lysozyme or C^{14} hemoglobin solutions and rinsed once with water, were divided into three segments termed the tip, the mid, and the basal portions. The portions were then frozen in isopentane cooled with liquid nitrogen, dehydrated in methyl alcohol at −30° to −40° C, transferred to toluene, paraffin infiltrated, sectioned, and mounted on glass slides. The sections were deparaffinized and radioautographs were made using the liquid emulsion technique (13). Exposures of 3, 7, and 12 days were used. After developing, the tissue was stained with Azure B. It is important to remember that in the liquid emulsion type radioautograph, the emulsion is 30 to 40 μ thick, and activity is seen as a track of from 4 to 14 grains in length. Only tracks indicate the site of activity—scattered individual grains, which in some preparations may be numerous, do not. The nature of the tracks produced by C^{14} can be seen in figure 3.

Titrations of excised roots were performed as follows: One gram of roots was suspended in 100 ml water in a beaker and the suspension was quickly brought to pH 4 with hydrochloric acid. Stirring was done by a magnetic bar which was activated from below. The pH remained constant for about 40 seconds before starting the titrations. The suspensions were titrated with a motor driven microburette to pH 8 with KOH or $Ca(OH)_2$ and the change of pH was followed simultaneously with a recording pH meter equipped with shielded electrodes. Data were also obtained for titrations of acid-in-water blanks.

A test for efficiency of stirring during the rapid titration of roots, by stopping and starting flow from the burette showed an instant response from the pH meter. No effort was made to exclude air during the titrations.

Results

Rate of Absorption of Proteins by Excised Roots: *Lysozyme.* In a typical experiment 2 g samples of washed and centrifuge-drained roots were suspended in either 10 ml of M/1,000 KH_2PO_4 of pH 5.1 or in M/1,000 KH_2PO_4 containing 0.349 mg/ml of lysozyme. The difference in optical density (at 2,800 Å) of the two supernatant solutions after a given time of mixing solution and roots is a measure of the amount of nonabsorbed enzyme. That is, material absorbing at 2,800 Å escapes from excised roots into dilute buffer at any given pH and the optical density of this material, which was found to be independent of the presence of lysozyme, must be subtracted from that due to the administered protein remaining in solution in order to obtain the true optical density of the protein solution in contact with the roots, (D). The initial optical density of 10 ml of lysozyme was also measured (D_1).

Data in table I are given by way of illustration and a sample calculation is as follows: The optical density of lysozyme is 2.64/mg/ml (14). The initial concentration of lysozyme, C_1, was $D_1/2.64 = 0.349$ mg/ml. After 30 minutes mixing the uptake of lysozyme by roots was 0.46 mg/g roots, i.e.,

$$\left[\frac{0.920/2.64 - (0.778 - 0.098)/2.64}{2 \text{ g}}\right] \times 10 \text{ ml} = 0.46 \text{ mg/g}$$

It is seen (table I) that the uptake is essentially complete after 10 minutes at this initial concentration level. If the initial concentration is as high as 1.32 mg/ml (fig 1A), the uptake continues slowly and linearly after 30 minutes as far as observed, namely for 2 hours. Extrapolation of the linear portions of

Table I

Absorption of Lysozyme from 10 ml of M/1,000 Phosphate Buffer (pH 5.1) by 2 g Excised Barley Roots

Content of bottle	Time mixing, min	D_{2800} of Supernatant	Absorption, mg lysozyme/g roots
Roots + lysozyme	2	0.845	0.34
" "	4	0.825	0.39
" "	10	0.772	0.47
" "	30	0.778	0.46
" "	60	0.790	0.43
" "	120	0.758	0.48
Roots + buffer	2	0.104	
" "	4	0.109	
" "	10	0.098	
" "	30	0.098	
" "	60	0.099	
" "	120	0.092	
Lysozyme	...	0.920	

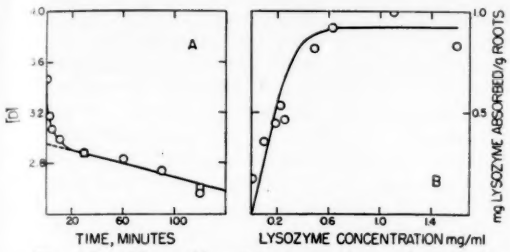

FIG. 1A. Absorption of lysozyme by excised barley roots at pH 5.2 as a function of time: 0, 25 ml bottles; □, 65 ml bottles. Ordinate = [D] of external solution.

FIG. 1B. Uptake of readily absorbable lysozyme by barley roots.

plots of such rate data back to zero time of exposure gives an estimate of the amounts of lysozyme, m, which we define as those amounts rapidly sorbed by roots (7).

The amount, for an initial concentration of 1.32 mg lysozyme per ml, as determined by back extrapolation to zero time, (fig 1A) is 1.0 mg lysozyme per g roots and the corresponding concentration of lysozyme in the surrounding solution (C), was 1.11 mg/ml. A plot of this datum along with other available points gives the isotherm of figure 1B. The maximum amount of readily sorbed lysozyme is seen by inspection to be about 0.93 mg lysozyme per gram of excised roots. As much as 1.72 mg/g roots of lysozyme is taken up with (C) equal to 1.42 mg/ml after 115 minutes, however. The uptake of lysozyme by barley roots was checked by an independent chemical method which confirmed the optical procedure.

Using 65 ml bottles the ratio of air to root plus solution was increased from about one-to-one to over four-to-one. No difference in rates of uptake of lysozyme by roots was detected in the presence of more oxygen. Excised roots which were cut up in order to increase the number of cut ends by a factor of three showed no increase in rate of uptake of lysozyme. Incidentally, roots which had been stored in a damp cloth overnight at 5° C and used 24 hours later showed the same uptake of lysozyme as fresh roots under otherwise identical conditions.

Hemoglobin. Data for the rate of uptake of hemoglobin by roots showed a trend similar to that in table I for lysozyme, at initial concentrations as high as 1.7 mg/ml, except that 25 to 30 minutes were required to reach equilibrium.

AMOUNTS OF PROTEINS ABSORBED BY EXCISED ROOTS: *Lysozyme.* As we have seen from the isotherm (fig 1B), the maximum amount of lysozyme which readily diffuses into roots is about 0.93 mg/g. A total uptake as high as nearly double this figure has been observed from a concentrated lysozyme solution after a contact of 2 hours duration.

Lysozyme-impregnated roots are not very subject to elution of lysozyme by a wash with water or M/1,000 phosphate buffer. Roots which had taken up the larger amount of lysozyme (1.72 mg/g) were tested, however, for the amount of protein which could be eluted with M/1 ethylamine hydrochloride of pH 5.7. Based on optical density readings at 2,800 Å, with corrections for readings from control roots without absorbed lysozyme, about half the absorbed protein was eluted from enzyme-impregnated roots during 10 minutes of mixing. About 90 per cent of the absorbed lysozyme can be eluted in 30 minutes.

Hemoglobin. The uptake of hemoglobin on roots, calculated from the rate data by the extrapolation procedure described above for lysozyme is summarized in table II. The isothermal data show no sign of leveling off to a maximum amount at the concentrations used and the weight of hemoglobin taken up can be greater than that of lysozyme.

TABLE II

UPTAKE OF HUMAN HEMOGLOBIN BY EXCISED BARLEY ROOTS IN M/100 KH_2PO_4 BUFFER, PH 5

PROTEIN CONC* IN AMBIENT BUFFER MG/ML	ABSORPTION IN MG PROTEIN/G ROOTS
0.55	1.09
1.05	1.86
1.31	2.01

* D_{2800}/mg/ml = 1.72

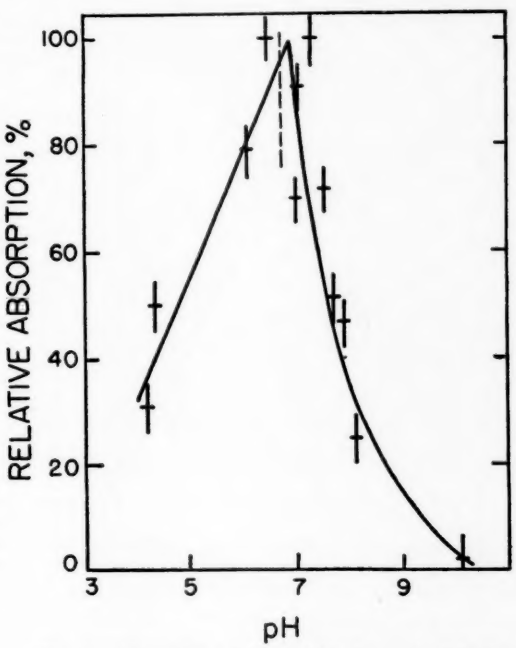

FIG. 2. Absorption of hemoglobin by excised barley roots as a function of pH. The dotted vertical line is at the isoelectric point of hemoglobin.

Since roots are supposed to be negatively charged (6) and hemoglobin is amphoteric, it is of interest to study the influence of pH on the uptake of hemoglobin by roots. Accordingly the relative amounts of hemoglobin taken up were observed in a series of 0.03 M phosphate buffer containing a constant initial amount of hemoglobin. The results are plotted in figure 2. Above and below the isoelectric point of hemoglobin the uptake falls to relatively small values.

At least a third of the absorbed hemoglobin at pH 5.7 can be eluted with ethylamine buffer; extracted roots still show the color of absorbed hemoglobin.

SITE OF UPTAKE OF PROTEINS IN ROOTS: As will be discussed below, the amounts of protein which can be taken up by barley roots exceed that expected for the adsorption of a monomolecular layer on the visible external surface of the roots. An effort was made to find hemoglobin in hand cut cross sections of roots by means of the blue color observable on mixing benzidine with hemoglobin and sodium perborate in glacial acetic acid. Root sections without hemoglobin gave some blue coloration in the cortex and an evolution of a deeper blue coloration in the presence of absorbed hemoglobin was not certain. Therefore we resorted to radiography by means of C^{14} labeled proteins.

Radioautographs obtained with intact or excised roots that had been placed in the C^{14}-lysozyme and C^{14}-hemoglobin showed that both compounds readily penetrated the root. The general distribution of the C^{14}-lysozyme can be seen in figures 3 and 5.

The anatomy of the barley roots has been described

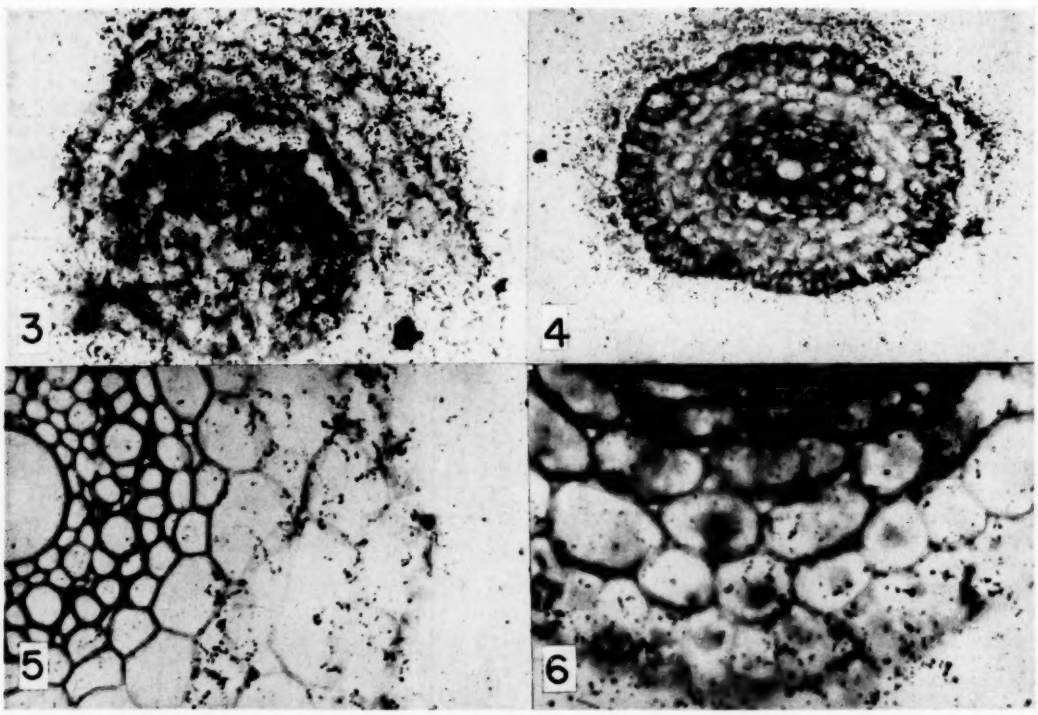

FIG. 3. Radioautograph of barley root after being placed in C^{14}-lysozyme for ¾ hour. The activity is indicated by tracks of silver grains. Two tracks that are particularly clear in this photograph are indicated by arrows. Numerous tracks can be seen throughout the section. × 60.

FIG. 4. Radioautograph of barley root after being in C^{14}-lysozyme for ¾ hour. This section was close to the tip of the root. Tracks can be seen distributed throughout the tissue. × 100.

FIG. 5. Radioautograph of barley root after being in C^{14}-lysozyme for ¾ hour. This is a section from a mature portion of the root composed of highly vacuolate cells. The tracks can be seen originating from the cytoplasm and the walls of the cells of the cortex and epidermis. Little if any activity can be seen in the vascular tissue. × 160.

FIG. 6. Radioautograph of barley root after being in C^{14}-hemoglobin for ½ hour. The tracks can be seen originating in the wall, cytoplasm, and surrounding the nucleus (N). × 250.

by Jackson (11). The sections shown in figure 3 and figure 4 are from an apical region of the root where the cells are developing rapidly. In both figure 3, which is of C^{14}-lysozyme, and figure 4, which is C^{14}-hemoglobin, activity can be seen between and in the cells throughout the apical region. The section illustrated in figure 5 is from a mature portion of the root and is a radioautograph of C^{14}-lysozyme. Here the activity is primarily concentrated between and in the cells of the epidermis and cortex. The cells of the stele show little or no activity.

In both the developing and mature cells the C^{14}-lysozyme and C^{14}-hemoglobin are found in the cell wall, on the cell surface, in the cytoplasm, and surrounding the nucleus. This can be seen for C^{14}-lysozyme in figure 4 and for C^{14}-hemoglobin in figure 6. The proteins were labeled with C^{14}-glycine and the possibility exists that the C^{14}-glycine was split from the proteins at the cell membrane and moved into the cell as a free amino acid. To test this possibility roots were placed in C^{14}-glycine for the same length of time as in the protein solutions, and radioautographs were made in the same manner as with the C^{14}-proteins. The same level of radioactivity was used. The tracks originating from the cytoplasm and nuclei of the cells were then counted in similar apical regions of roots placed in C^{14}-glycine, C^{14}-hemoglobin, and C^{14}-lysozyme. The results are presented in table III. The data indicate that C^{14}-glycine is about equally incorporated by the nucleus and the cytoplasm while the C^{14} in the cells placed in the protein solutions is found primarily in the cytoplasm. This marked difference in distribution strongly argues against the possibility that the labeled glycine of the proteins is moving into the cells as a free amino acid. If this were the case the distribution would not be expected to show such a marked difference.

The distribution of the labeled protein within the cell can be seen in greater detail in radioautographs using H^3-lysozyme and H^3-ribonuclease. The beta particle emitted by tritium is much lower in energy than that emitted by carbon 14. The energy is so low that in radioautographs with tritium by the liquid emulsion procedure the tracks are only one or two silver grains in length. Hence, localization is very precise. Radioautographs of roots placed in H^3-lysozyme are shown in figures 7 and 8 and for H^3-ribonuclease in figures 9 and 10. For both enzymes the localization is the same and the same as the two C^{14}-proteins: in the cell wall, on the cell surface, in the cytoplasm, and surrounding the nucleus. The H^3-ribonuclease is, in addition, sometimes found in the nucleolus. We have confirmed the observation of Brachet (3) that ribonuclease is active inside the living cell causing marked reduction in RNA in the cytoplasm and nucleolus as shown by specific cytochemical reactions. This would indicate that ribonuclease is entering the cell either intact or nearly so, since 4/5ths of the molecule is essential for activity (1). Roots were placed in tritiated water of the same activity as the labeled protein and for the same length of time. In the normal procedure the roots are dehydrated before paraffin infiltration and sectioning; thus, if little or no exchange occurred between the tritiated water and the substances composing the cells of the root, no activity should be visible in the radioautographs. Under the identical conditions that produced the radioautographs shown in figures 7 to 10, no activity was demonstrated in the roots placed in tritiated water.

The identical distribution of H^3-ribonuclease, H^3-lysozyme, C^{14}-lysozyme, and C^{14}-hemoglobin within the cell together with the markedly different distribution of C^{14}-glycine and the negative result with tritiated water, all suggest that the proteins are entering the cells intact, possibly through a similar mechanism.

TITRATION BEHAVIOR OF BARLEY ROOTS: In order to compare the exchange capacity of roots with protein uptake, a neutral equivalent of roots was determined. Titrations were carried out, generally from pH 4 to 8, both rapidly (1–1.4 min) and slowly (several mins). With potassium hydroxide, 0.15 ± 0.05 meq was required per 100 g fresh weight of roots during a rapid titration and 0.5 ± 0.05/100 g for a slow titration (8–9 min), when the titration was begun in less than a minute after the pH had been rapidly adjusted to pH 4. The total consumption of base during a long titration was the same starting from either pH 3 or 4, and the roots show little or no acidic property from pH 3 to 5. That is, the root seems to titrate significantly only above about pH 5. During these rapid titrations the ionic strength of the suspension was very low. Increasing the ionic strength to a nearly constant value during the rapid titration by the presence of 0.01 *N* KCl did not influence the base consumption beyond the experimental uncertainty of the titrations. Equivalent titres of $Ca(OH)_2$ and KOH were required to reach pH 8 during slow titrations.

TABLE III

ORIGIN OF TRACKS IN CELLS OF ROOTS PLACED IN C^{14}-GLYCINE, C^{14}-LYSOZYME, AND C^{14}-HEMOGLOBIN

COMPOUND	NO. CELLS COUNTED	NO. TRACKS FROM	
		NUCLEUS	CYTOPLASM
C^{14}-Glycine	1,050	2,240	2,163
C^{14}-Hemoglobin	900	45	892
C^{14}-Lysozyme	950	56	743

Discussion

We have seen that both lysozyme and hemoglobin are absorbed by barley roots at a pH near 5. We do not know the maximum amount of lysozyme which can be taken up, but as much as 1.72 mg per g roots has been observed at pH 5.5 At this pH the lysozyme molecule has a net charge of + 12.6 (14). For a molecular weight of 15,000 for this protein, this uptake amounts to

$$\frac{12.6 \times 1.72 \text{ mg/g} \times 100 \text{ g} \times 10^{-3} \text{ g/mg} \times 10^{3}}{15{,}000} = 0.14 \text{ meq/100 g fresh wt}$$

If we assume that cation exchange plays a role in the uptake process we may compare this uptake with the titratable hydrogen ion from roots, namely 0.15 to 0.5 meq/100 g, as stated above, and the previously reported exchange capacity of roots, namely 0.45 to 1.5 meq/100 g for barium and ammonium (22). The idea of an exchange reaction taking place during the uptake of proteins by roots is supported by figure 2 which is similar to that for the adsorption of hemoglobin by montmorillonite wherein ion exchange has actually been observed (18). The explanation for the uptake as a function of pH is the same for both the clay and roots, namely that as the pH is increased above pH 5 the net negative charge on the root increases without changing sign but the net charge on the protein decreases, becoming less positive, and eventually becomes negative. Thus the tendency of the root to attract more protein with increasing pH is counteracted by the tendency of the protein to become negative which results in a maximum in figure 2. The corresponding experiment with roots and lysozyme has not been carried out because of the very high isoelectric point of lysozyme. The same con-

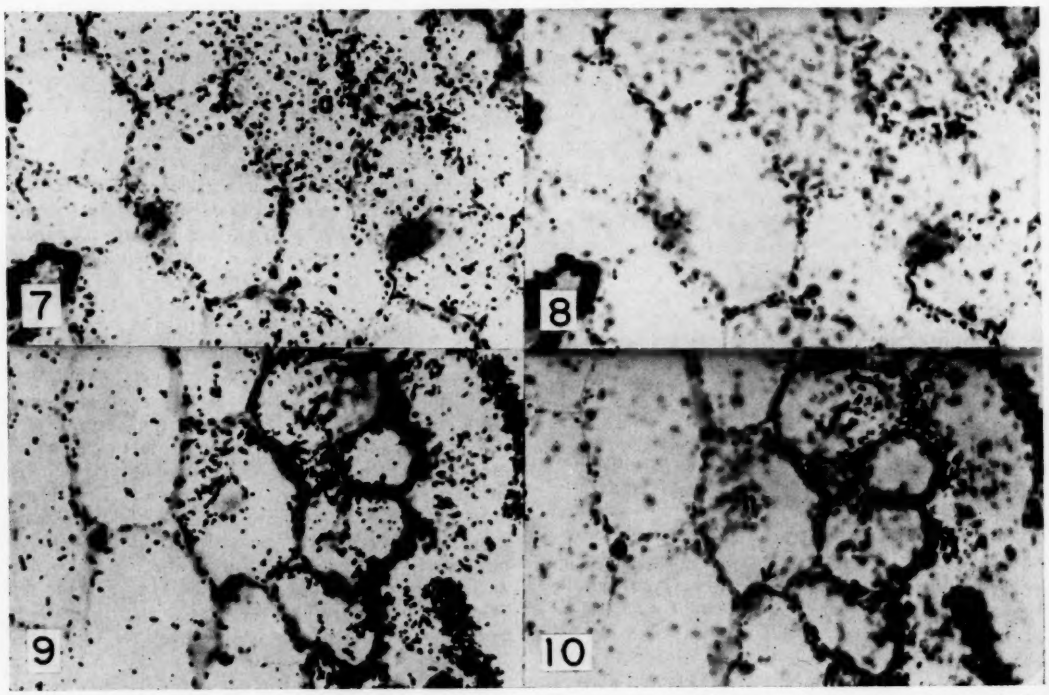

Fig. 7. Radioautograph of barley root after being in H^3-lysozyme for 2/3 hour. In this photomicrograph the cytoplasm at the end of one cell (a) is in focus and exposed silver grains indicate that the activity is in the cytoplasm. Activity can be seen in the cytoplasm of other cells, surrounding nuclei, and associated with the cell wall and cell membrane. No activity is visible in the vacuoles. × 400.

Fig. 8. Same cells as figure 7 but here the nuclei and walls are in focus. The silver can be seen surrounding the nuclei and associated with the cell surface. × 400.

Fig. 9. Radioautograph of barley root after being in H^3-RNA'ase for 2/3 hour. In this photomicrograph, the cytoplasm of the central cell is in focus. Activity is again seen to be localized in the cytoplasm and associated with the cell surface. No activity is visible in the vacuoles. × 400.

Fig. 10. Same cells as figure 2 but here the nuclei and walls are in focus. The H^3-RNA'ase can be seen surrounding the nuclei (N) and associated with the cell surface (arrow). × 400.

clusion applies to lysozyme and clay, however (18). The relatively low uptake of hemoglobin at pH 4 may be accounted for by competition of hydrogen ions for the negative absorption sites in the root at this low pH.

Although we already have evidence that the proteins penetrate the root, it is of interest to consider the geometry and kinetics of the problem. From the uptake isotherm, figure 1B, we see that 0.93 mg lysozyme is readily taken up by a gram of roots. In separate trials it was found that the total length of a gram of excised roots varied from 547 to 614 cm and the average diameter from 0.470 to 0.475 mm, giving minimal external surface areas of from 82 to 91 cm^2/g. Now 0.93 mg lysozyme as a close-packed monolayer can cover an area of about 3×10^3 cm^2 so that the observable surface area of a root could accommodate only about 1/30 the lysozyme absorbed.

On the other hand, the root, with its nearly 85 per cent water content is not just soaking up protein molecules entirely as if the protein were penetrating into a bag of water through a permeable membrane, as can be seen from the following analysis of a datum in table I. Two grams of barley roots were placed in 10 ml of lysozyme solution containing 3.49 mg lysozyme. After 10 minutes, 0.94 lysozyme had been taken up by 2 g roots containing ca. 1.7 g water. If simple diffusion had taken place the amount taken up would have been only

$$0.349 \text{ mg/ml} \times 10 \text{ ml}/(10 + 1.7 \text{ ml}) \times 1.7 \text{ ml} = 0.51 \text{ mg}$$

assuming that all the water was available for dissolved protein.

At low values of (C) the actual uptake has been found to be as high as 6.3 times the amount expected by simple diffusion. Thus some absorption sites, probably ion exchange sites, are doubtless involved in the uptake of lysozyme by roots, in addition to any possible free space solution uptake (5). Further, at high values of (C), e.g., 1.55 mg/ml, the uptake can fall below, namely, 0.86 of that expected by simple diffusion, indicating that most binding sites are saturated and that only a portion of the water content is available for diffusion. The fact that the sorbed protein may be partly eluted by ethylammonium ion in solution but not by water alone also shows ion exchange.

It is noteworthy that a molecule the size of hemoglobin, with a diameter of about 53 Å, penetrates so readily into intact or excised roots[3]. The radioautographs reveal that most of each protein sorbed by the roots is localized between cells and in the cell walls. Clearly some of the protein enters the root cells. For the following reasons it seems highly probable that the C^{14}-labeled proteins are not being broken down by root tissue into readily diffusible labeled glycine peptides. A. Native lysozyme is resistant to proteolytic hydrolysis (15) and is not hydrolyzed by barley roots in 2 hours. B. The evidence about ovalbumin (M.W. = 45,000) quoted by Starkey (20) and mentioned above. C. It has been amply demonstrated from the influence of ribonuclease (M.W. = 15,000) on growth and cell structures that this enzyme penetrates the cells of onion root tips (2). Our data show that both randomly labeled H^3-ribonuclease and H^3-lysozyme enter the cell and show similar patterns of distribution. Moreover, the patterns of distribution for H^3-lysozyme and C^{14}-lysozyme are identical. D. The distributions of C^{14}-glycine and C^{14}-lysozyme in the cell are markedly different.

We have no mechanisms to suggest for the uptake of proteins by plant cells. Ribonuclease and lysozyme (4) are known to be taken up intact by animal cells through the process of pinocytosis [see Holter (10) for a recent review of the literature]. The association of H^3-ribonuclease (fig 8) and H^3-lysozyme (fig 10) with the surface of the cytoplasm together with recent electron photomicrographs of plant cells (8) showing the presence of invagination in the surface of the cytoplasm suggests that pinocytosis may occur in plant cells.

A possible significance of the capacity for roots to absorb macromolecules in terms of plant nutrition in soil has been discussed by Starkey (20). Brachet (2) pointed out that ". . . those textbooks that state in a dogmatic way that proteins are unable to cross the cell membrane barrier have not only been wrong but harmful, because they have hampered scientific progress". It would seem desirable to examine the implications of these observations in plant nutrition, plant virology and in terms of mechanisms by which plant pathogens exert harmful effects with their exoenzyme systems.

Summary

Both lysozyme and hemoglobin can penetrate negatively charged barley roots below the isoelectric points of the proteins to the extent of more than 1 mg per g fresh roots. Ion exchange and free space are probably both involved in the uptake process, but if the root and the protein are both sufficiently highly negatively charged the uptake is nil. The penetration of C^{14}-hemoglobin, H^3-lysozyme, and H^3-ribonuclease into the root was followed by radioautographs. All of these proteins appear to enter the intercellular spaces and the cell walls. Some of each sorbed protein was found associated with the surface of the cytoplasm, in the cytoplasm, and surrounding the nucleus.

[3] We have analyzed the rate of uptake of the lysozyme and hemoglobin by roots in terms of equations for diffusion from a solution into a cylinder by assuming that the root is roughly uniform. The apparent diffusion constants obtained are about half the known values for free diffusion in water. Although our radioautographic results show that uptake of proteins by the roots is not uniform, the calculated constants are consistent with the notion that uptake is rapid. Such calculations reveal nothing about the mechanism of movement of proteins into the roots, however.

Acknowledgments

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Literature Cited

1. Anfinsen, C. B. 1957. Structural basis of ribonuclease activity. Fed. Proc. 16: 783–791.
2. Brachet, J. 1957. Biochemical Cytology. New York, Academic Press.
3. Brachet, J. 1954. Effects of ribonuclease on the metabolism of living root tip cells. Nature. 174: 876.
4. Brandt, P. W. 1958. A study of the mechanism of pinocytosis. Exp. Res. 15: 300–313.
5. Briggs, G. E. and R. N. Robertson. 1957. Apparent free space. Ann. Rev. Plant Physiol. 8: 11–30.
6. Burström, H. 1941. Studies of the carbohydrate nutrition of roots. Ann. Agricultural College of Sweden 9: 264–284.
7. Butler, G. W. 1953. Ion uptake by young wheat plants. II. The apparent free space of wheat roots. Physiol. Plantarum 10: 617–635.
8. Buvat, R. 1958. Recherches sur les infrastructures du cytoplasme, dans les cellules du meristeme apical, des ebauches foliaires et des feuilles developpées d'*Elodea canadensis*. Ann. sci. Nat. Bot. II^{e} serie, 19: 121–161.
9. Estermann, E. F. and A. D. McLaren. 1959. Stimulation of bacterial proteolysis by adsorbents. Jour. Soil Sci. 10: 64–78.
10. Holter, H. 1959. Pinocytosis. Intern. Rev. Cytol. 8: 481–504.
11. Jackson, V. G. 1922. Anatomical structure of the roots of barley. Ann. Bot. 36: 21–39.
12. Jacobson, J., R. Overstreet, R. M. Carlson, and J. A. Chastain. 1957. The effect of pH and temperature on the absorption of potassium and bromide by barley roots. Plant Physiol. 32: 658–662.
13. Jensen, W. 1957. The incorporation of C^{14}-adenine and C^{14}-phenylalanine by developing root-tip cells. Proc. Nat. Acad. Sci. A. S. 43: 1038–1046.
14. McLaren, A. D. 1954. The adsorption and reactions of enzymes and proteins on Kaolinite. I. Jour. Phys. Chem. 58: 129–137.
15. McLaren, A. D. 1954. The adsorption and reactions of enzymes and proteins on Kaolinite. II. Soil Sci. Soc. Amer. Proc. 18: 170–174.
16. McLaren, A. D. 1957. Concerning the pH dependence of enzyme reactions on cells, particulates, and in solution. Science 125: 697.
17. McLaren, A. D. and K. L. Babcock. 1959. Some characteristics of enzyme reactions at surfaces. In: Subcellular Particles, Symposium Monograph of the American Physiological Society, T. Hayashi, ed. Pp. 23–26. Ronald Press, N. Y.
18. McLaren, A. D., G. H. Peterson, and I. Barshad. 1958. The adsorption and reactions of enzymes and proteins on clay minerals. Soil Sci. Soc. Amer. Proc. 22: 239–244.
19. Margen, S. and H. Tarver. 1957. The deiodination of proteins labeled with I^{131}. Ann. New York Acad. Sci. 70: 49–54.
20. Starkey, R. L. 1958. Interrelations between microorganisms and plant roots in the rhizosphere. Bacteriol. Rev. 22: 154–172.
21. Steinberg, D., M. Vaughan, C. B. Anfinsen, and J. Gorry. 1957. Preparation of tritiated proteins by the Wilzbach Method. Science 126: 447–448.
22. Williams, D. E. and N. T. Coleman. 1950. Cation exchange properties of plant root surfaces. Plant and Soil 11: 243–256.

ACTION OF GIBBERELLIC ACID ON LETTUCE SEED GERMINATION[1, 2]

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Several workers have shown that gibberellic acid (GA_3) promotes the germination of seeds which ordinarily require light (6, 10, 13, 14, 16, 17, 23, 28, 30). This phenomenon raises several fundamental questions as to the mechanism of light-controlled germination: A: Does light simply act by releasing a gibberellin in the seed, B: Does the GA_3 substitute for light by bringing about chemically a reaction which is normally photochemical, or C: Does the GA_3 bring about some quite other type of growth process which independently leads to germination? The following experiments are an attempt to provide answers to these questions.

MATERIALS AND METHODS

The seeds used for germination studies were those of *Lactuca sativa* L. var. Grand Rapids (Breck's Co., Boston, Mass.). For some of the gibberellin extraction work the seeds were supplied by Pieters Wheeler Seed Co., Gilroy, Cal. Since germination in the dark was found to be gradually induced even in dry seeds, when stored at room temperature and humidity (cf. 5), special precautions were taken as to the time of purchase of the seeds and their storage. Seeds purchased at the end of January, 1959, and kept in the cold room at 4° C thereafter, showed less than 5% germination in darkness. These were used for all quantitative experiments. They were taken out of the cold room as needed, allowing the shortest possible time of exposure to room temperature and humidity.

The ordinary method in which seeds are placed on wet blotter or filter paper gave markedly fluctuating results. For this reason the present work was carried out by shaking the seeds in solution without filter paper. Throughout the periods of imbibition and germination, the seeds were placed in 5 cm Petri dishes with the solution, and gently shaken on a rotating shaker in the dark at 25° C. Three milliliters of solution was added to each Petri dish, when 100 seeds were used, and 2 ml when 50 seeds or less were used. With this method, the seeds are treated with a precisely measured volume of water, thoroughly wetted and aerated; correspondingly the results have been much more uniform, although the overall averages, especially for the first 2 hours, are not much changed by the filter paper.

For the introduction of solutions by injection, 25 seeds whose fruit-coats had been removed were imbibed for 15 minutes on double layers of wet filter paper wrapped around two sheets of slide glass placed in a 9 cm Petri dish, and the solution was injected from the cotyledon end with a fine needle (Vita 30, Vita Needle Co., Needham, Mass.) in between the endosperm and the embryo. The endosperm layer (the innermost covering of the seed) separated from the embryo and swelled due to the liquid injected. The criterion of success in injection was that the liquid should penetrate as far as the radicle tip of the seed. According to this criterion, the reliability of the technique was more than 80%. To complete this procedure on 25 seeds required 7 to 10 minutes. Injection was carried out at 4° C under the green safe-lamp, and the seeds were then transferred to darkness at 25° C for germination.

Gibberellic acid (GA_3) was used as a stock 100 ppm solution in distilled water, and dilutions were prepared from this just before use.

The red light source was a 100 watt incandescent lamp filtered through a Corning Signal Red glass filter (50% transmission at 630 mμ), and the source for far-red light was a General Electric 150 watt reflector flood lamp filtered through the Corning glass filter No. 7–69 (2600) (50% transmission at 742 mμ). Both these filters are of the steep cut-off type. An interference filter with peak transmission at 735 mμ was also employed for a few experiments, but because of its small size was found inconvenient to use. The lamps were placed 25 cm above the level of the seeds. Necessary manipulations which required light were carried out under a green safe-lamp (20 watt white fluorescent lamp wrapped by double layers each of yellow and green cellophanes). In a few cases the germination occurred in an atypical manner; these are denoted by numbers in parentheses in the tables.

RESULTS

I. NORMAL COURSE OF GERMINATION. Preliminary experiments were carried out to find the amount of moisture which must be absorbed to allow germination under the conditions employed. For this purpose the fresh weight of the seeds was determined at various periods after the beginning of imbibition at 25° C. One group of seeds was induced to germinate with 2 minutes exposure to red light at 1.5 hours after the beginning of dark imbibition, and the other groups were kept in darkness throughout. For comparison with the imbibition data, the extent of germination which had been induced by 1 minute irradiation with red light at various times after the beginning of imbibition was also determined. The results are shown in figure 1; each reading was the average of two runs.

Curves A and B show that light does not stimulate the uptake of water by the seeds, but shortly after visible germination begins a marked increase in

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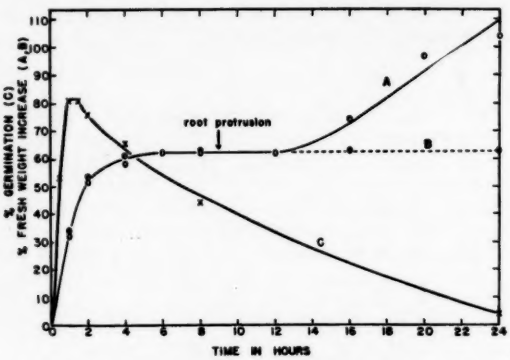


FIG. 1. Percent increase of fresh weight (A and B) during the course of imbibition and germination. A: Seeds induced to germinate with 2 minutes red light at 1.5 hours after the beginning of imbibition. B: Seeds kept in darkness. C: Effect of 1 minute irradiation with red light, given at indicated times, on germination examined 48 hours after the light treatment.

weight occurs; this parallels the elongation of the seedling (cf. fig 4). Although 1 minute of exposure to the red light is not sufficient to induce full germination at any period of imbibition, it is clear that the seeds are most sensitive, showing a single peak, at about 1¼ hours from the beginning of imbibition, when water uptake is still taking place actively and the water content of the seeds has reached about 40 % of their original weight (figure 1C). This observation indicates that seeds need not be in the fully turgid state for maximum germination to be induced.

As is well known, exposure of the seeds to far-red light inhibits germination (2, 4, 8, 9). However, Borthwick et al (2) showed that seeds which had been induced to germinate by red light escaped the inhibitory effect of far-red irradiation, if the far-red was given a sufficiently long time after red irradiation. Figure 2 shows a detailed study of this effect. The seeds were exposed for 2 minutes to red light after 1.5 hours (Curve A) or 8 hours (Curve B) from the beginning of dark imbibition, then given 2 minutes far-red light at various intervals after the red treatment. An average of two readings is plotted in this figure.

The curves of figures 2A and 2B agree fairly well in general shape, except that figure 2B tends to show a longer lag period, slower rate of escape, and less germination at the maximum level. After the first sign of root penetration, the curves reach the plateau, i.e., complete escape from far-red inhibition (see arrows). In comparison with figure 2, Borthwick et al found that the initial lag period continued up to 3 hours and their seeds became gradually less sensitive up to about 15 hours (fig 9 of ref 2). The markedly slower timing is probably due to differences in the experimental method as discussed below. However, their curve essentially coincides in its shape with those reported here.

As shown in figure 1C, at 1.5 hours after dark imbibition the seeds are in a state of maximum sensitivity and by 8 hours they have become much less responsive to red light. Some fluctuations in the data of figure 2B may reflect the gradual decrease in sensitivity due to the long imbibition period, which by the end of the experiment totals 24 hours.

II. EFFECT OF GIBBERELLIC ACID. That lettuce seeds are as responsive to GA_3 in the dark[3] as has been reported in the literature (6, 10, 13, 14, 17) is shown in figure 3, which includes data of two experiments. In one case the concentration of GA_3 needed for 50 % germination in the dark is approximately 28 ppm and maximum germination is obtained at 60 ppm; in the second experiment 50 % germination was produced by 20 ppm and 100 % at between 50 and 100 ppm. In both cases controls in water gave less than 1 % germination.

When the seeds are shaken with the solution, only a small amount of GA_3 may be taken up, so that the actual sensitivity is doubtless higher than that shown above. For this reason the action of GA_3 was also tested by direct injection into the seeds. The method of injection has been described above. In table I the results of three series of experiments are summarized. Unfortunately with this method many control seeds (injected with water) germinate, but the results are still fairly clear; maximum germination can be obtained with a concentration as low as 1 ppm. This point will be referred to in section V below.

It is well known that GA_3 has a marked effect on elongation of shoots (see 3, 26). However, Evenari et al (6), who made measurements of the hypocotyls and roots of lettuce seedlings 72 hours from the start of soaking, concluded that GA_3 had no effect on the

[3] Minimum green light was used briefly for the manipulations.

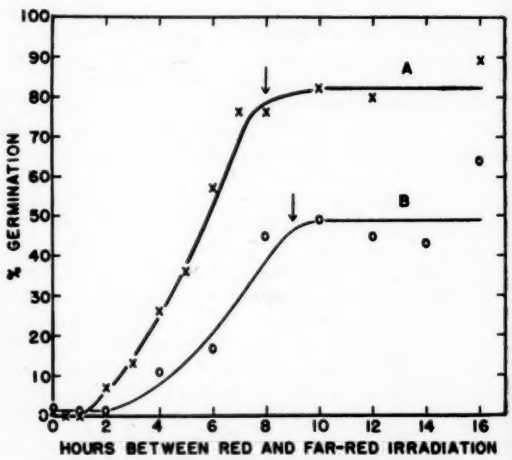
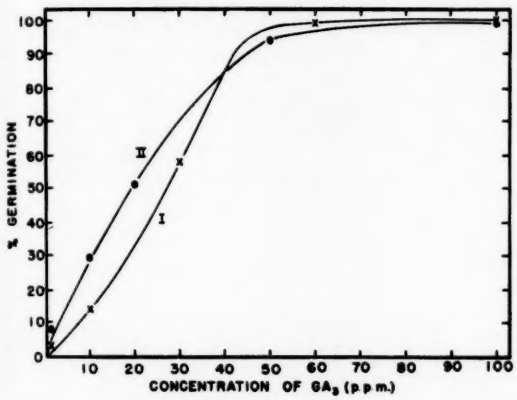
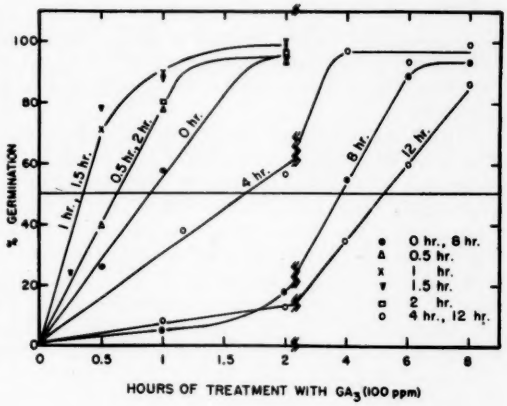


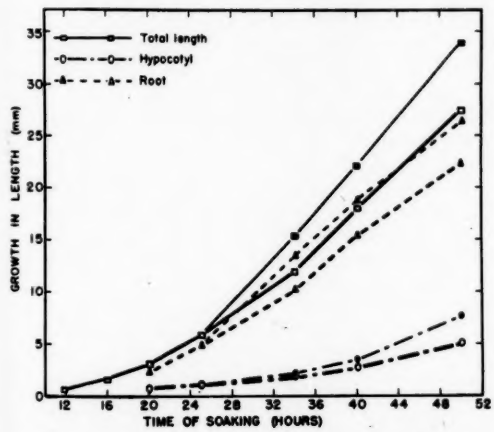
FIG. 2. Effect of far-red irradiation at various periods after the seeds have been induced to germinate with red. Red light given after 1.5 hours (A) or 8 hours (B) from the beginning of imbibition. Exposures to red and far-red 2 minutes in each case. The arrows indicate the time of the first sign of root protrusion.

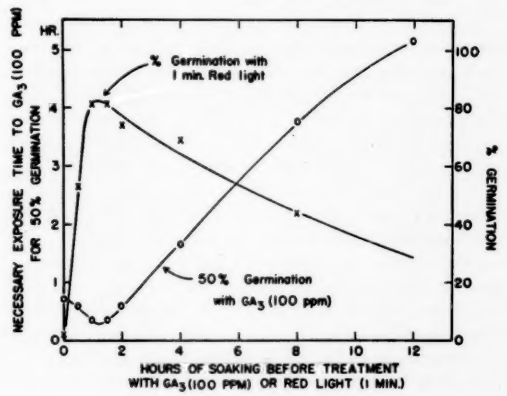
growth of hypocotyls or roots. Their data, nevertheless, do actually show an increase of 10 to 20 % in the length of the hypocotyls. The timing of their measurements, furthermore, may have been late enough so that these two parts of the seeds had already reached maximum growth. Since a possible mechanism of GA_3 action on the germination of lettuce seeds is that it promotes the initial rate of growth of these parts, this point seemed worthy of reinvestigation. For this purpose, 50 seeds were soaked in GA_3 solution (100 ppm) in darkness for various periods of time between 12 and 48 hours, then the lengths of hypocotyls and roots of all germinated seedlings were measured. As control, a group of seeds were allowed to imbibe in the dark in water, then given 2 minutes exposure to red light at 1.5 hours after dark imbibition, and measured as above. The results are shown in figure 4.

The first sign of visible germination of both light-induced and GA_3-treated seeds takes place after almost the same period of dark imbibition, or the GA_3-treated seeds may be a little later, i.e., about nine hours from the beginning of soaking. It is, however, difficult to measure the length of the elongated radicles just after their emergence. The rates of growth of hypocotyls and roots of treated and control seeds appear not to differ much at first. In both organs the gibberellin treatments show appreciable increase in growth rate only after 16 hours from the first sign of visible germination, i.e., 25 hours after the begin-








HR.
% Germination with 1 min. Red light
50% Germination with GA3 (100 ppm)
NECESSARY EXPOSURE TIME TO GA3 (100 PPM) FOR 50% GERMINATION
% GERMINATION
HOURS OF SOAKING BEFORE TREATMENT WITH GA3 (100 PPM) OR RED LIGHT (1 MIN.)

FIGS. 3–6. Action of gibberellin on germination.

FIG. 3 (*top left*). Effect of GA_3 at various concentrations on germination in darkness. I, experiment of May 30, 1959; II, experiment of July 9, 1959.

FIG. 4 (*lower left*). Growth in length of hypocotyl and root. Germination induced either by 2 minutes red light given 1.5 hours from the beginning of soaking or by 100 ppm GA_3 given throughout the period of imbibition and germination. Open symbols (thick lines) light-induced seeds; closed ones (thin lines) GA_3 treated.

FIG. 5 (*top right*). Germination of lettuce seed as influenced by duration of treatment with GA_3 (100 ppm) begun at various times after presoaking in water. Hours given on each curve (and inset) indicate the length of time presoaked in water; abscissa the duration of the GA_3 treatment.

FIG. 6 (*lower right*) Necessary exposure time to 100 ppm GA_3 to cause 50 % germination (*left ordinate*), and per cent germination with 1 minute red light (*right ordinate*), after various periods of presoaking in water.

TABLE I

EFFECT OF GIBBERELLIN IN VARIOUS CONCENTRATIONS WHEN INJECTED INTO GRAND RAPIDS LETTUCE SEED*

Conc of GA_3 (ppm)	% Germination		
	Expt 1	Expt 2	Expt 3
0	26 + (2)	36	24
0.1	40 + (4)	...	28 + (4)
0.3	...	...	32 + (8)
1.0	84 + (6)	98	66 + (6)
10	...	94 + (6)	...
30	...	98 + (2)	...

* Each reading the average of 50 seeds.
Numbers given in brackets represent atypical germination.

ning of soaking (fig 4). Thus it seems difficult to ascribe the germination caused by GA_3 to any promotion of the initial growth of emerging hypocotyls and roots.

This conclusion was confirmed by using the modified procedure described in section III, in which 50 seeds were treated only for 2 hours with GA_3 solution (100 ppm) after 1.5 hours of water presoaking in darkness, and then rinsed three times in water. Thus the external GA_3 was removed long before visible germination began. After 50 hours from the beginning of soaking, the lengths of hypocotyls were: in red light treated 5.1 mm (s.d., 1.62) and in GA_3 treated 4.7 mm (s.d., 1.50), while the lengths of the roots were 21.6 mm (s.d., 4.10) and 23.3 mm (s.d., 5.06), respectively. It is evident that the differences between the two groups are not statistically significant ($t = 1.27$ for hypocotyl and $t = 1.82$ for roots, hence $P < 0.05$ for both) and therefore that the gibberellin, when applied only before the time of visible germination, does not increase the elongation of the seedling.

It may be added that at no period after the beginning of water imbibition did GA_3 (100 ppm) increase the uptake of the solution by the seeds, prior to visible germination, above that by the water controls.

It has been shown that seeds whose endosperm coverings have been removed are perfectly capable of elongating their radicles in the dark (1, 11). The responsiveness of the seed to light is thus determined by the integrity of the endosperm layer; this phenomenon makes possible a clear separation between the true germination process and simple elongation of the radicle (11). The above data, therefore, suggest that the action of GA_3 on seed germination, like that of red light, is exerted on the true germination process, rather than by promoting the growth of the radicle. Furthermore, the data also offer evidence that GA_3 so applied does not persist in the seed after germination (cf. below).

III. TIME RELATIONS OF ACTION OF GA_3. According to Borthwick et al (2), when seeds are induced to germinate by red light, a previous imbibition in water of about 12 hours is needed for the light to have its maximum effect (fig 1 of ref 2). In our own experiments, however, the corresponding time, as shown in figure 1, is only about 1¼ hours. The optimum time for treatment with GA_3 was therefore investigated. Evenari et al (6) have concluded that 10 hours' soaking in gibberellin solution (2.9×10^{-5} M = 10 ppm), followed by transfer to water, gave more complete germination than soaking throughout the period in the gibberellin solution. In their experiments, however, both the quality of the seeds and the experimental method were somewhat different from those employed in our study.

A hundred seeds were first soaked in darkness in 3 ml of distilled water for various periods of time between 0 and 12 hours, then quickly dried on filter paper, and transferred to GA_3 solution, 100 ppm, for specified periods between 15 minutes and 8 hours. The drying and transfer to GA_3 solution necessitated exposure to the green safe-light for 4 to 5 minutes. After each period of soaking in GA_3, the seeds were immediately washed three times with distilled water and quickly blotted dry between filter papers.

Care was taken not to press or roll the seeds, as this might have promoted germination. The seeds were then placed in Petri dishes, each containing 3 ml of distilled water, and allowed to germinate. Forty-eight hours after transfer of the seeds to GA_3 solution, the germination was examined. Groups of seeds were prepared as controls in the same manner as indicated above, except that they were transferred for 1.5 hours to distilled water instead of GA_3 solution. The results (fig 5) show in general that the later the gibberellin is applied the less effective it is, i.e., the longer the seeds must be soaked in it to achieve the same effect. However, in the first 2 hours the trend is in the opposite direction, the gibberellin being more effective than if applied at the start. The curves for 0.5 and 2 hours, and those for 1 and 1.5 hours, overlap; evidently the most effective time to treat with GA_3 lies between 1 and 1.5 hours. At this time 50 % germination can be achieved by soaking in the solution for 20 minutes.

In order to show this more clearly, the necessary exposure time to GA_3 required to induce 50 % germination is plotted against the duration of presoaking in water before exposure to GA_3 (fig 6). The greatest sensitivity for the induction of germination by the gibberellin occurs after about 1¼ hours of presoaking in water. For comparison, curve C of figure 1, which shows the percentage germination with 1 minute of red light exposure after various periods of soaking, is plotted alongside in figure 6. As pointed out already, the seeds are most sensitive to red light after about 1¼ hours of imbibition in darkness. Thus *GA_3 and red light are both at their most effective stage after exactly the same time from the beginning of the dark imbibition of water.*

In a repeat of this experiment the results showed essentially the same pattern of curves and timing. The only difference was that the percentage of germination obtained was a little lower in every case, i.e., the slopes of the curves of figure 5 were a little less.

TABLE II

EFFECTS OF FAR-RED LIGHT ON GERMINATION OF GRAND RAPIDS LETTUCE SEEDS, AS FUNCTION OF TREATMENT WITH RED LIGHT OR WITH GA_3 (100 PPM)

TREATMENT	% GERMINATION
Red (2 min)	92
Far-red (5 min)	1
Red (2 min), then far-red (5 min)	0
Far-red (5 min), then red (2 min)	91
GA_3 (2 hrs)	93
Far-red (5 min), then GA_3 (2 hrs)	93
GA_3 (2 hrs), then far-red (5 min)	93
Water, dark control	2

IV. INTERACTION BETWEEN GIBBERELLIN AND FAR-RED LIGHT. It could be concluded from the above that the action of GA_3 is to substitute for red light. If this were true, its effect should be reversible by far-red, as is the effect of red light. However, previous reports indicate either that this is not the case (13, 17), that it is only partially reversible (6, 14), or in one case (10) that it is indeed reversible. However, in this last case, the seeds were held at 22° C for 12 hours and then transferred to 37°C; the germination was low and the effect of GA_3 was partially reversed by red light also. Thus, it is not easy to draw a firm conclusion from all these data because the extent of germination in the dark varies so greatly between the seeds and the procedures used by different workers. In order to re-examine the effect of far-red on germination caused by gibberellin, the following two experiments were carried out using the same technique as described above; i.e., the seeds were treated only for a short time with GA_3 (100 ppm) and not throughout the period of imbibition and germination.

In the first experiment, seeds were exposed to GA_3 for 2 hours after 1.5 hours of presoaking in water, (the optimum time, as shown in fig 6) then washed three times with water, and transferred to water. A generous dosage of far-red light (5 min) was given immediately before or after the exposure to GA_3. Table II shows the results, each reading being the average of 200 seeds. It is clear that, although the GA_3 treatment is capable of inducing germination to the full extent, its effect is not reversed at all by far-red light. This finding is further strengthened by the fact that in the same experiments, under the same conditions, the action of red light is fully reversed.

Haber and Tolbert (10), however, showed that the GA_3 effect was completely reversed by far-red irradiation for 20 minutes, although, as stated above, their experimental methods were different from ours. It seemed nevertheless possible that the results in table II were obtained because the exposure to far-red light was too short in comparison with the long treatment with GA_3. Therefore in a second experiment the seeds were treated with GA_3 for a sufficient period to bring about only 50 % germination (30 min or 1.5 hrs according to the length of pre-soaking time in water) and far-red light was given immediately before or after the GA_3 treatment for 10 or 50 minutes (the latter being given as five 10-min exposures evenly spread through the 1.5 hr period). The results, shown in table III, indicate that when far-red light was given *before* the GA_3 treatment, the light reduced germination by 37 to 38 %, and when given after the GA_3 by 20 %. In a third set of experiments five 10-minute exposures to far-red light were again given immediately before or after the GA_3 treatment (30 min after 1.5 hrs of presoaking in water), and the results showed essentially the same pattern as above; i.e., far-red treatment reduced germination by 63 % when given before and only by 31 % when given after GA_3. It may therefore be concluded that far-red light is capable of desensitizing the seeds to the GA_3 treatment but not of reversing completely the GA_3 action, even when given for 50 minutes. Normally 1 minute of far-red under our conditions is sufficient for complete reversal of the germination caused by red light.

These results show that far-red, especially in large dosages, reduces the sensitivity of seeds to the subsequent action of gibberellin. It is indeed actually more effective in reducing the germination if it is

TABLE III

EFFECTS OF FAR-RED LIGHT ON GERMINATION OF GRAND RAPIDS LETTUCE SEEDS TREATED WITH GA_3 (100 PPM). GA_3 TREATMENT ADJUSTED IN TIMING TO BRING ABOUT 50 % GERMINATION

TREATMENT	% GERMINATION	INHIBITION OF GERMINATION %
Far-red (10 min)	1	...
GA_3 (30 min)	45	...
Far-red (10 min), then GA_3 (30 min)	28	38
GA_3 (30 min), then far-red (10 min)	36	20
Far-red (50 min)[1]	1	...
GA_3 (1.5 hrs)[2]	46	...
Far-red (50 min)[1], then GA_3 (1.5 hrs)[2]	29 + (1)	37
Dark, water control	0 + (1)	...

Seeds were given GA_3 or far-red treatment at 1.5 hours after the beginning of soaking unless otherwise mentioned.

[1] Far-red light was given as five 10-minutes exposures during 1.5 hour period after 1.5 hours of dark presoaking in water.

[2] GA_3 was given after 3 hours from the beginning of dark imbibition for 1.5 hours.

( ) indicates atypical germination.

given *before* the GA_3 treatment than when given after. It is clear, however, that far-red does not in any real sense *reverse* the action of the gibberellin. The answer to the problem posed at the outset of this section is therefore that gibberellin does not simply substitute for red light.

V. Attempts to Identify a Gibberellin-like Substance as Product of Photoreaction. An alternative approach to the problem of the interrelationship between the effects of gibberellin and of red light is to determine if gibberellin is produced by the photo-reaction as the end-product of the process. In this case the external application of GA_3 would simply promote the germination irreversibly. It is now well established that gibberellin-like substances occur in higher plants (18, 19, 20, 21, 22, 24, 25, 27, 29) and both GA_1 and GA_5 have actually been isolated from higher plant material (18, 27, 29). Although these findings are not necessarily connected with the action of light, they obviously increase the probability of a photoproduction of gibberellin.

From the results presented in table I, calculations were made before experimentation as to the maximum yield of gibberellin-like substances which should be expected in the extracts if red light acts by releasing such material in the seed. Since about 0.002 ml of a 1 ppm solution of GA_3 causes maximal germination, then, allowing for only 20 % recovery in the extracts, 10 gm seeds should be required to yield 4 μg gibberellin. If we further consider that the gibberellin acts only at a specific locus, and only 20 % of that injected reaches this locus, then the expected yield would drop further to 0.8 μg. Even such an amount is large in comparison with the known responses of test plants. Therefore, 10 gm seeds were extracted with acetone-water (24) for 10 hours at 34° C 2 or 8 hours after the light treatment (10 min exposure to white light at 1.5 hrs after the beginning of dark imbibition), and the dried extract was extracted with 1 ml of water (including 0.05 % Tween 20), of which 0.1 and 0.5 ml were applied to dwarf-1 mutants of corn. As controls, dry seeds and dark-imbibed seeds were also extracted in the same manner. In none of the extracts, however, could the presence of gibberellin-like substances be detected. The bioassay responds clearly to 0.001 μg. of GA_3 (24). Furthermore, paper chromatography was carried out on a strip of Whatman No. 1 filter paper, but no indication of any spot fluorescing in ultraviolet light after treatment with concentrated sulfuric acid was shown with two solvent systems employed.

In the meantime, extraction of a gibberellin-like substance from dry lettuce seeds was reported (A. Lang. Personal communication of unpublished work by S. Blumenthal-Goldschmidt and A Lang.) (20). A second series of experiments were, therefore, conducted using 500 gm of seeds. The seeds were given 10 minutes of white light at 1.5 hours after the beginning of dark imbibition (the optimum time, as shown in fig 1), and 2 hours later were extracted with acetone-water (1:1), and the extract fractionated with ethyl acetate (29). Dry seeds and dark-imbibed seeds were extracted in the same way, and the extracts of all three were again tested on the dwarf-1 mutant. The final dried residue of the ethyl acetate reextract was dissolved in 10 ml of distilled water (containing 0.05 % Tween 80), and 0.1 ml of this was used for assay. The lengths of the leaf-sheaths are given in table IV. The total elongation of the first leaf during the 7 days was: dwarf control 2.0 cm, dry seeds 2.8 cm, dark imbibed 2.0 cm, light treated 2.9 cm. The data show a small positive reaction in the assay, but the amount was not increased by light treatment over that present in the dry seed.

According to Neely (21) the best values for calibration of the test are given by adding together the lengths of the two leaf sheaths; these data are given in the last column of the table. It may be deduced

Table IV

Final Lengths (cm) of 1st and 2nd, Leaf Sheaths of Dwarf-1 Corn, Treated With Extract from Lettuce Seeds, and of Controls *

Solution	Amt. applied per d-1 plant	1st Leaf sheath	2nd Leaf sheath	Sum
Extract from 1,000 gm dry seeds in 10 ml	0.1 ml	3.25cm	3.10cm	6.35cm
Extract from 500 gm dark-imbibed seeds in 10 ml	0.1 ml	1.90	2.35	4.25
Extract from 500 gm light-induced seeds in 10 ml	0.1 ml	2.10	2.35	4.45
GA_3 (Crystalline)	0.0001 μg	2.10	2.53	4.63
	0.001 μg	2.60	3.30	5.90
	0.01 μg	3.83	4.87	8.70
0.05 % Tween-80 water	0.1 ml	1.90	2.43	4.33
Tall sibling plants	...	5.97	8.05	14.02

* Time of measurement, for first leaf sheath 7 days, for second leaf sheath 10 days, after treatment. Nutrient solution XL 36, white light 1450 ft-c, 25° C. Each figure the mean of two or three plants.

from this calibration that 10 gm seeds yield only about 0.003 μg. (dry seeds) or 0.0002 μg. (light-induced seeds) instead of the calculated 0.8 μg.

Since the test solution might have contained injurious materials or growth inhibitors, it was thought that the growth promotion of the test plants might have been depressed. An attempt, therefore, was made to remove any inhibitor present by paper chromatography in a solvent (*n*-butanol: glacial acetic acid: water = 95: 5: 30) which separated the GA_3 spot (Rf = 0.80–0.81) from the rest. A section of paper chromatogram between Rf = 0.7 and Rf = 1.0 was reextracted with acetone-water (1: 1), the dried residue dissolved in Tween 80 as above, and again bioassayed. The very small yield of the extracted gibberellin-like substance from the control seeds was again not increased, but even somewhat decreased, by the light treatment.

The method employed by West and Phinney (29) leads to the isolation of GA_5 (Bean Factor II), to which dwarf-3 or -5 corn is more sensitive than dwarf-1. Furthermore, A. Lang (Personal communication of unpublished work by S. Blumenthal-Goldschmidt and A. Lang.) has informed us that the material extracted from dry lettuce seeds behaves rather like GA_5. Therefore a third series of experiments was carried out using dwarf-5 corn seedlings as assay plants. Lettuce seeds (30 gm) were extracted four times with acetone-water (1: 1) as above. After condensing each extract to about 2.5 ml, about 20% of it was paper-chromatographed with *n*-butanol: glacial acetic acid: water = 95: 5: 30 and the chromatograms cut into five equal sections according to the Rf value. Each section was eluted with 1: 1 acetone-water four times, the eluate dried completely, taken up into 1 ml water, and 0.1 ml of this bioassayed by applying 0.04 ml between the coleoptile and the first leaf, and 0.06 ml inside the unfolding first leaf, within a 12 hour period. Figure 7 shows the growth of the first and second leaves, and table V gives the final lengths of the leaf sheaths. These data show a definite growth promotion; the highest value at Rf = 0.8 — 1.0, slight activity at Rf = 0.6 — 0.8, and none at lower Rfs. However, again the light treatment clearly produced no increase in the amount of extracted gibberellin.

The calibration with a sample of pure GA_5 (obtained from Dr. J. MacMillan of Imperial Chemical Indus.) indicates that the maximum amount extracted (i.e., from the dark-imbibed seeds) corresponds to 0.002 μg of GA_5; there are, therefore, 0.03 μg per 10 gm seeds. This compares well with Murakami's figure (21) of 0.025 μg per 10 gm. It is about ten times as much as was shown in table IV using dwarf-1 corn. The amount is clearly not increased, but even decreased, by the light treatment.

From these results it is confirmed that Grand Rapids lettuce seeds contain a small amount of gibberellin-like substance whose biological activity is more like that of GA_5 than GA_3, but that *the amount of the substance in the extracts is not increased by light treatment.* This repeated failure to demonstrate any increase in the amount of the gibberellin-like substance in the seeds after exposure to light makes it improbable that light can act by releasing a gibberellin within the seed.

TABLE V

FINAL LENGTHS (CM) OF 1ST AND 2ND LEAF SHEATHS OF DWARF-5 CORN, TREATED WITH EXTRACT FROM LETTUCE SEEDS, AND OF CONTROL*

SOLUTION	RF VALUE	AMT APPLIED/ D-5 PLANT	1ST LEAF SHEATH	2ND LEAF SHEATH	SUM
Extract from 30 gm dark-imbibed seeds in 1 ml	0.0–0.2	0.1 ml	1.85cm	2.15cm	4.00cm
	0.2–0.4	0.1 ml	1.75	2.00	3.75
	0.4–0.6	0.1 ml	1.80	1.90	3.70
	0.6–0.8	0.1 ml	2.00	2.55	4.55
	0.8–1.0	0.1 ml	3.15	3.25	6.40
Extract from 30 gm light-induced seeds in 1 ml	0.0–0.2	0.1 ml	1.75	2.15	3.90
	0.2–0.4	0.1 ml	1.85	2.15	4.00
	0.4–0.6	0.1 ml	1.85	1.95	3.80
	0.6–0.8	0.1 ml	1.95	2.60	4.55
	0.8–1.0	0.1 ml	2.50	2.55	5.05
GA_5 (crystalline)	...	0.00001 μg	2.05	1.85	3.90
	...	0.0001 μg	2.55	2.40	4.95
	...	0.001 μg	2.70	2.95	5.65
	...	0.01 μg	3.40	4.20	7.60
0.05% Tween-80 water	...	0.1 ml	1.93	2.13	4.06
Tall sibling plants		...	4.57	6.20	10.77

*Nutrient solution XL 36, white light 1450 ft-c, 25° C. Each figure the mean of two or three plants.

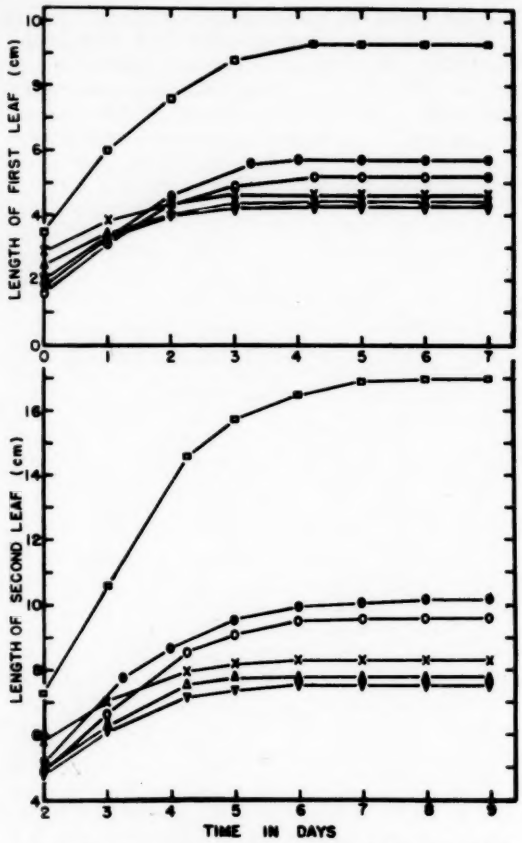


FIG. 7. Bioassay of extracts from light-treated and dark-imbibed seeds (30 gm each) using dwarf-5 corn seedling. Zones of chromatogram of Rf = 0.6 to 0.8 and 0.8 to 1.0 eluted with acetone-water (1:1), and 10% of each eluate applied to individual plants. (Zones of lower Rf not shown, because they caused no growth promotion.) —□—□—, tall sibling plant; —×—×—, dwarf-5 control treated with Tween-80 water; —○—○—, eluate (Rf = 0.8–1.0) from light-treated seeds; —●—●—, eluate (Rf = 0.8–1.0) from dark-imbibed seeds; —△—△—, eluate (Rf = 0.6–0.8) from light-treated seeds; —▽—▽—, eluate (Rf = 0.6–0.8) from dark-imbibed seeds. Above: growth of the first leaf. Below: growth of the second leaf.

Discussion

IMPORTANCE OF FULL WATER SUPPLY. It has been known for some time that lettuce seeds vary in their sensitivity to red and far-red light according to the preceding period of dark imbibition. Borthwick et al (2) reported that in their system the germination reached a near-maximum value when Grand Rapids seeds were exposed at from 12 to 20 hours after the beginning of imbibition. Evenari and Neumann (4) reported that with 500 ft-c-minutes irradiation the maximum germination was obtained when the seeds were exposed after 16 minutes at 22° C or 30 minutes at 26° C, while with shorter periods of irradiation the maximum was reached only after 8 hours' imbibition. Flint (8), using the Big Boston variety and 600 ft-c-minutes' illumination found maximum germination (90.5%) when the illumination came after soaking 100 minutes at 25° C. Neither of the above time schedules for var. Grand Rapids agrees with the results presented in this paper, in which the highest sensitivity to red light falls at about 1¼ hours. The discrepancies may in part be due to the different amounts of water available to the seeds. In preliminary experiments in which moist filter paper was used as wetting medium, the time relations at the start were similar to figure 1C, but divergent later. Figure 8 shows a typical result with double layers of filter paper and 1 ml of distilled water in 5 cm Petri dishes; the largest change from figure 1C is at 4 and 8 hours, where the presence of GA_3 maintains the germination at high levels.

The presence of filter paper also changes the effectiveness of far-red in the presence of GA_3, for in figure 8 far-red light appears partially to reverse the GA_3 effect. In this experiment exposure was only for 1 minute, while in table II, with 5 minutes exposure to the same light, but without filter paper, there was no reversal. Even 50 minutes exposure (table III) gave much less reversal than in figure 8. It is possible that GA_3 becomes adsorbed on the paper so that the amount entering the seeds is reduced, but subsequently it is slowly released, giving the seeds a delayed treatment. The method used in the present experiments, without filter paper, thus has several advantages.

WATER UPTAKE AND SENSITIVITY OF SEED. It is extremely interesting that maximum germination resulted from exactly the same duration of water imbibition prior to the treatments, whether the germination was caused by red light or by GA_3 (fig 6). A similar finding has recently been reported by Yamaki et al (30) for light-sensitive tobacco seeds. This observation indicates that the metabolism of the lettuce seed is most ready for induction when its water content reaches about 40%. At this time saturation with water is still far from complete. However, some experiments in which the seed was separated into fruit-coat, cotyledons, and hypocotyl + root, indicate that the curves of figures 1A and 1B mainly represent the behavior of the cotyledons, and the hypocotyl + root unit in fact reaches its maximum water content before the 2nd hour. The maximum sensitivity of the seed to red light may, therefore, be attained as soon as the uptake of water by the hypocotyl + root region has been completed. Presumably the water uptake makes the seeds ready for the induction of germination by initiating their metabolism.

A POSSIBLE MECHANISM OF GA_3 ACTION ON LETTUCE SEED GERMINATION. The light sensitivity of the seed evidently depends upon the integrity of the

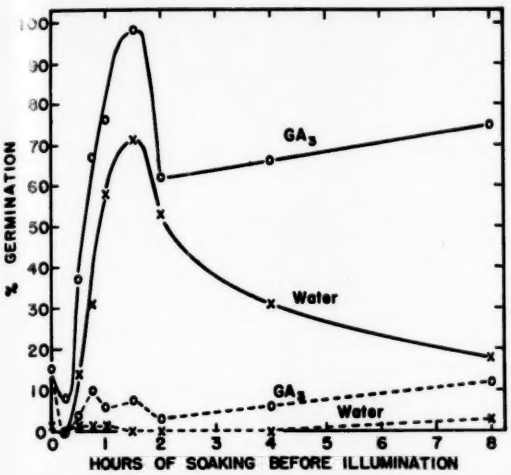


FIG. 8. Effect of 1 minute red (*solid lines*) or 1 minute far-red (*dotted lines*) after various periods of presoaking on germination of lettuce seeds imbibed in 1 ml of GA_3 solution (100 ppm) or water in presence of double layers of filter paper.

endosperm layer, the innermost covering of the seed, since it disappears when this is removed (1, 11). But the photosensitive site resides in the hypocotyl region, where active elongation takes place (11, 12). The actual elongation of the radicle of seeds in which the endosperm no longer restricts germination appears not to be appreciably influenced by light (11). Klein and Preiss (15) have shown too that the photosensitivity of the seeds does not change even when they are turned over in the interval between red and far-red irradiation.

These facts suggest that the postulated pigment system is in the hypocotyl, rather than in the endosperm, and that on red light treatment this pigment activates one or more chemical reactions which lead to breaking the mechanically restricting endosperm layer.

That GA_3 so closely mimics the effect of red light in its action and timing strongly suggests that it acts at the same site as red light, namely in the embryonic hypocotyl. Yamaki et al (30) reported that substances which induce germination of tobacco seeds in darkness diffuse out from leaf discs which have been illuminated with red light, and that one of these substances has the same Rf value as that of the gibberellins on chromatograms developed with several different solvent systems. Although this observation strongly suggests that a gibberellin is produced in tobacco seeds by red light, our extractions have failed repeatedly to demonstrate that even a trace of gibberellin is produced by light treatment of lettuce seeds. This applies both to GA_3 and to GA_5. One must conclude, therefore, that it is most improbable that light induces germination of lettuce seeds by liberating a gibberellin. Haber and Tolbert (10) have come to a similar conclusion.

The most reasonable explanation of the action of gibberellic acid, therefore, is that it somehow initiates, independently of light, one of the series of chemical reactions leading to germination, thus producing the same final product for breaking the mechanical restriction of the endosperm layer. This would account both for the non-reversibility of gibberellin action by far-red and for the identity of timing of its effect with that of red light. Thus, of the three possible alternatives listed in the Introduction, the second one appears nearest to the truth.

SUMMARY

I. The effect of gibberellic acid (GA_3) on the germination of Grand Rapids lettuce seeds has been compared with the action of red light, in the hope of elucidating the mode of action of these factors.

II. The work was carried out by shaking the seeds directly with a GA_3 solution or water throughout the period of imbibition and germination. This method was found to yield more uniform and more repeatable results than the ordinary one using moist filter paper.

III. During the course of imbibition, the seeds gradually become sensitive to red light, and reach their maximum sensitivity 1¼ hours after the beginning of imbibition, when water uptake is still not complete; thereafter the sensitivity to red light gradually decreases.

IV. In seeds which have been induced to germinate by red light, the inhibitory effect of far-red light gradually decreases with increasing time interval between red and far-red treatments. Far-red light no longer inhibits the germination process after the first sign of visible germination occurs (8–9 hrs after red light treatment at 25° C).

V. GA_3 at 60 ppm induces maximal germination; germination is almost linearly proportional to concentration below this level. However, if the solution is injected directly into the seed, concentrations as low as 1 ppm are fully effective.

VI. The action of GA_3 is not to control the initial rate of growth of the hypocotyl and the root, since this rate is increased only at about 16 hours after the first sign of visible germination.

VII. The time of greatest sensitivity for the induction of germination by gibberellin occurs after about 1¼ hours of presoaking in water; this timing is identical with the most sensitive period of the seeds to red light.

VIII. Far-red light does not inhibit the germination of seeds which have been treated with GA_3 so as to produce 90 % germination. If the time of GA_3 treatment is adjusted to give only 50 % germination, and the far-red exposure increased, then far-red does partially inhibit, but this inhibition is exerted to the same extent whether the exposure is made *before or after* the GA_3 is applied. Thus, heavy doses of far-red desensitize the seeds to gibberellin.

IX. Repeated attempts to detect a gibberellin as the end-product of the action of light in the seeds failed completely, whether the assay was for GA_3 or

GA_3. Small amounts of gibberellin were detected in the seeds, but the quantity was not increased by a light dosage sufficient to cause maximum germination.

X. It is deduced that GA_3 acts by initiating one of the chemical reactions which result from the light reaction, so that its end product is the same as that produced by light.

Acknowledgments

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Literature Cited

1. Borthwick, H. A. and W. W. Robbins. 1928. Lettuce seed and its germination. Hilgardia 3: 275–304.
2. Borthwick, H. A., S. B. Hendricks, E. H. Toole, and V. K. Toole. 1954. Action of light on lettuce seed germination. Bot. Gaz. 115: 205–225.
3. Brian, P. W. 1959. Effects of gibberellins on plant growth and development. Biol. Rev. 34: 37–84.
4. Evenari, M. and G. Neumann. 1953. The germination of lettuce seeds. III. The effect of light on germination. Bull. Res. Counc. Israel 3: 136–145.
5. Evenari, M. and G. Neumann. 1953. The germination of lettuce seeds. IV. The influence of relative humidity of the air on light effect and germination. Palestine Jour. Bot., Jerusalem Ser., 6: 96–100.
6. Evenari, M., G. Neumann, S. Blumenthal-Goldschmidt, A. M. Mayer, and A. Poljakoff-Mayber. 1958. The influence of gibberellic acid and kinetin on germination and seedling growth of lettuce. Bull. Res. Counc. Israel 6D: 65–72.
7. Flint, L. H. 1934. Light in relation to dormancy and germination in lettuce seed. Science 80: 38–40.
8. Flint, L. H. and E. D. McAlister. 1935. Wave lengths of radiation in the visible spectrum inhibiting the germination of light-sensitive lettuce seed. Smithsonian. Misc. Coll. 94(5): 1–11.
9. Flint, L. H. and E. D. McAlister. 1937. Wavelengths of radiation in the visible spectrum promoting the germination of light-sensitive lettuce seed. Smithsonian Misc. Coll. 96 (2): 1–8.
10. Haber, A. H., and N. E. Tolbert. 1959. Effects of gibberellic acid, kinetin, and light on the germination of lettuce seed. In: Photoperiodism and Related Phenomena in Plants and Animals. Amer. Assoc. for Advancement of Science, Washington, D. C.
11. Ikuma, H. and K. V. Thimann. 1958. The mechanism of germination in light-sensitive lettuce seed. Plant Physiol. 33 suppl.: xxiv.
12. Ikuma, H. and K. V. Thimann. 1959. The photosensitive site in lettuce seeds. Science 130: 568–569.
13. Kahn, A., J. A. Goss, and D. E. Smith. 1957. Effect of gibberellin on germination of lettuce seeds. Science 125: 645–646.
14. Kahn, A. 1960. Promotion of lettuce seed germination by gibberellin. Plant Physiol. 35: 333–339.
15. Klein, S. and J. W. Preiss. 1958. Reversibility of the red-far-red reaction by irradiation at different sites. Nature 181: 200–201.
16. Kribben, F. J. 1957. Die Abkürzung der Samenruhe bei Arabidopsis durch Gibberellinsäure. Naturwiss. 44: 313.
17. Lona, F. 1956. L'acido gibberellico determina la germinazione dei semi di *Lactuca scariola* in fase di scoto-inhibizione. L'Ateneo Parmense. 27 (4): 641–644.
18. MacMillan, J. and P. J. Suter. 1958. The occurrence of gibberellin A_1 in higher plants: Isolation from the seed of runner bean (*Phaseolus multiflorus*). Naturwiss. 45: 46.
19. Murakami, Y. 1959. A paper chromatographic survey of gibberellins and auxines in immature seeds of leguminous plants. Bot. Mag. Tokyo 72: 36–43.
20. Murakami, Y. 1959. The occurrence of gibberellins in mature dry seeds. Bot. Mag. Tokyo 72: 438–442.
21. Neely, P. M. 1959. The development and use of a bioassay for gibberellins. Ph. D. Thesis, University of California at Los Angeles.
22. Nitsch, J. P. 1958. Présence de gibbérellines dans l'albumen immature du Pommier. Bull. soc. bot. France 105: 479–482.
23. Ogawara, K. and K. Ono. 1957. Tabako-shushi no hikari-hatsuga ni oyobosu gibberellin no eikyo (the effect of gibberellin on the light-induced germination of tobacco seeds). (In Japanese). Pp. 9–10. Abstr. 1st Meeting Jap. Gibberellin Res. Assoc.
24. Phinney, B. O., C. A. West, M. Ritzel, and P. M. Neely. 1957. Evidence for "gibberellin-like" substances from flowering plants. Proc. Nat. Acad. Sci. 43: 398–404.
25. Radley, M. 1958. The distribution of substances similar to gibberellic acid in higher plants. Ann. Bot., N. S. 22: 297–307.
26. Stowe, B. B. and T. Yamaki. 1957. The history and physiological action of the gibberellins. Ann. Rev. Plant Physiol. 8: 181–216.
27. Sumiki, Y. Occurrence of GA_1 in fruits of Japanese citrus. Proc. Intern. Cong. Plant Growth Substances, Yonkers, N. Y. (in press).
28. Toole, V. K. and H. M. Cathy. 1959. Germination of Grand Rapids Lettuce and *Lepidium virginicum* seeds as affected by gibberellin. Plant Physiol. 34 suppl: xvi.
29. West, C. A. and B. O. Phinney. 1959. Gibberellins from flowering plants. I. Isolation and properties of a gibberellin from *Phaseolus vulgaris* L. Jour. Amer. Chem. Soc. 81: 2424–2427.
30. Yamaki, T., T. Hashimoto, T. Ishii, and M. Yamada. 1958. Some physiological effects of gibberellin on seed germination, leaf expansion, dehydration of mitochondria, and probable formation of gibberellin in leaf. Pp. 51–52. Abstr. 2nd Meeting Jap. Gibberellin Res. Assoc.

BINDING OF ENZYMES TO AVENA COLEOPTILE CELL WALLS[1,2]

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It has been shown that the auxin, indoleacetic acid (IAA), exerts a marked influence on the pectic metabolism in growing cell walls of the Avena coleoptile (12). The methyl ester group in pectic substances is known to be derived, in part at least, from the methyl group of methionine (21, 22). Through the use of methionine-$C^{14}H_3$, it was shown that IAA markedly increases the rate of formation of the methyl groups in the cell wall pectic material without detectably changing the degree to which the pectic substances are esterified (12).

It has been suggested that the effect of IAA in promoting the elongation of cells is mediated through an effect on pectinesterase (PE). Some confusion exists as to whether IAA increases or effectively inhibits PE action. For example, Yoda (23) observed a parallelism between the effect of auxin on PE activity and on the water absorption of etiolated pea stems, and advanced the suggestion that the cell wall becomes elongated through a plasticization by PE activated by IAA. On the other hand, Glasziou (7, 8, 9) finds that IAA promotes the binding of PE to cell wall preparations from tobacco pith and artichoke tubers. He suggests that the immobilization of PE, by binding to the cell wall in vivo, favors methylation of the pectates of the cell wall and hence increases cell wall plasticity. However, he found the optimal concentration of IAA for binding of PE to cell wall preparations to be 10^{-9} M, a concentration far below that required to elicit the maximal rate of growth response. Conversely, Bryan and Newcomb (6) report that the PE activity of homogenates of tobacco pith is unaffected by IAA concentrations below 4×10^{-5} M but that concentrations of 1×10^{-4} M and higher completely inhibit the enzyme. They also observe that IAA increases the PE content of cultured tobacco pith just as it does the fresh weight.

The influence of IAA on PE activity and on the binding of PE to cell walls has therefore been reinvestigated to determine whether or not the effect of IAA in promoting the elongation of cells is mediated through an effect on PE.

MATERIALS AND METHODS

ENZYMES AND ASSAY METHODS: The PE was a relatively crude preparation from orange flavedo (15). It was dialyzed against 0.05 M $NaHSO_3$ at 5° C for several days, then against water until the sulfite had been removed. The preparation was then lyophilized. PE determinations were made by the standard procedure previously described (15). *a*-Chymotrypsin was prepared from chymotrypsinogen[5] recrystallized twice, dialyzed against water, and lyophilized. *a*-Chymotrypsin was assayed by a procedure similar to that for PE, with tyrosine ethyl ester as substrate (3).

A dialyzed and lyophilized preparation of the crystalline diisopropyl fluorophosphate inhibited *a*-chymotrypsin (10) was used in this study.

Crystalline pepsin prepared from commercial pepsin by the method of Northrop (19) was assayed for its rennet action (13).

Horseradish peroxidase[5] activity was measured by the method of Morris et al (18).

Nitrogen was determined with Nessler's reagent. Ammonium sulfate was used as a standard.

METHODS OF ANALYSIS: Pectic substance was measured by determining the uronic acid content using the pectinase-carbazole method of McComb and McCready (16) and McCready and McComb (17), expressing the pectic substance as anhydrouronic acid.

For determining methanol (methyl ester) content of cell wall preparations, 100 mg of dried samples was saponified with 4 ml of 1 *N* NaOH for 30 minutes at room temperature, and then 4.25 ml of 1 *N* HCl was added. The methanol so formed was distilled and determined by the method of Boos (5).

PREPARING AVENA COLEOPTILE CELL WALLS: Oat seeds of the variety Siegeshafer were germinated in Vermiculite moistened with distilled water, in stainless steel trays. The plants were grown at a temperature of 25° C and in low-intensity orange light until

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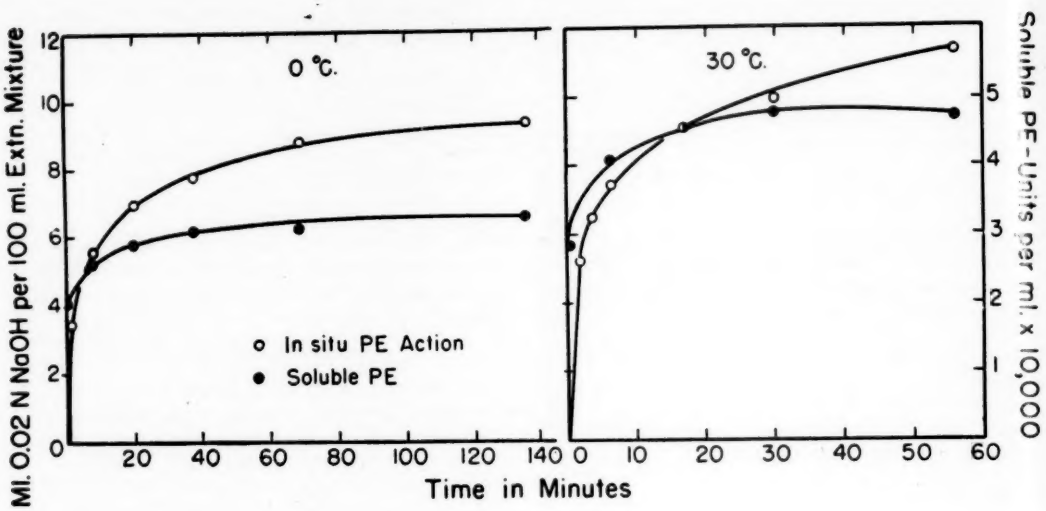
[2] Work conducted under a cooperative agreement at the California Institute of Technology.
[3] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.
[4] Division of Biology, California Institute of Technology.
[5] Purchased from the Worthington Biochemical Laboratory, Freehold, N. J.



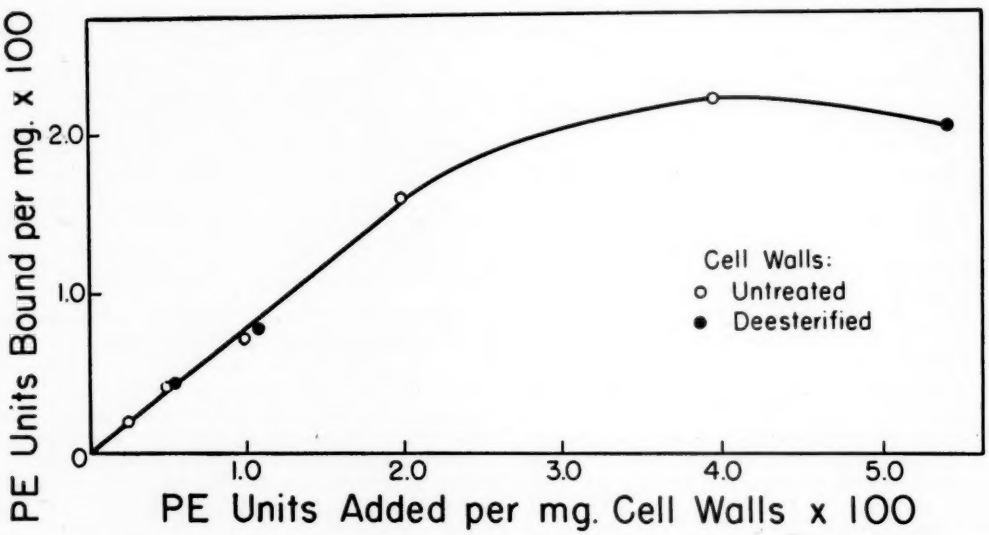


FIG. 1. PE action in situ and PE extraction at 0° C and 30° C: The action in situ was determined by measuring the amount of 0.02 *N* NaOH required to maintain pH 7.5 in a mixture of 50 ml of Avena coleoptile cell wall preparation plus 50 ml of H_2O and NaCl at a final concentration of 0.15 *N*. PE extraction was measured by periodic removal of aliquots which were then centrifuged and the PE activity of the supernatant determined.

FIG. 2. PE-binding capacity of untreated and deesterified cell walls: Weighed samples of partially purified PE were dissolved in 1 ml of cell wall suspensions at pH 5.6 (untreated contained 8.1 mg dry weight per ml and deesterified, 7.4 mg per ml) cooled to 0° C. After two hours the suspensions were centrifuged and aliquots of the supernatants were assayed for PE.

they had reached an average height of 3 cm (96 hrs). At this stage the seedlings were harvested.

Two methods of preparing cell walls were employed. One consisted of grinding the coleoptiles in an Omnimixer[6] at 0° C in four times their weight of water. The homogenate was centrifuged and the supernatant liquid decanted. The cell wall fraction was washed five times with water and finally suspended in water in a volume equal to twice the weight of the original coleoptiles. The pH of the suspension was 5.6.

Since PE bound to cell walls is inactive at pH values of 4.5 and lower (11), cell wall material was also prepared by grinding the coleoptiles in four times their weight of 0.15 M acetate buffer, pH 4.4 at 0° C. (PE assays of such homogenates, as well as the supernatant liquids obtained from centrifugation, showed that more than 90 % of the enzyme was still present in the cell walls.) Cell walls were then washed with water, and finally suspended in water as above. The pH of the final water suspension was 4.4.

Preparing Deesterified (PE-free) Cell Walls: Both the presence of salts and a pH of approximately 7.5 are known to be required for extraction of PE in orange tissue (15). The same conditions were also found to be necessary for extracting the enzyme from Avena coleoptile cell walls. The time course of the extraction of PE under these conditions and the simultaneous in situ PE action are given in figure 1 for temperatures of 0° and 30° C.

The major portion of the PE is extracted immediately upon adjustment of the pH of the extraction medium to 7.5. Further extraction of the enzyme occurs simultaneously with PE action in situ. These results appear to be in contrast to those obtained with orange cell walls. In the latter case, in situ PE action either preceded or occurred simultaneously with extraction of the enzyme. The results with orange suggest that the binding of PE to cell wall is similar to enzyme-substrate complex formation and that the enzyme is not extracted until the cell wall substrate has been hydrolyzed (11). Orange cell walls contain 39 % pectic substances (on a dry weight basis) of which 55 % is initially esterified (11) whereas coleoptile cell walls contain only 5.1 % pectice substances of which 43 % is esterified. Accordingly, less substrate is present for complexing in the latter cell walls and this may explain the difference in behavior of the two cell wall preparations.

Two types of deesterified Avena coleoptile cell wall preparations were made for enzyme binding studies; one preparation adjusted to pH 5.6 and the other to pH 4.4. For preparing the former, to 220 ml of a water-prepared cell wall suspension was added 1.93 g of NaCl and the suspension was cooled to 0° C, after which the pH was adjusted to 7.5 with 1 *N* NaOH. The suspension was stirred slowly and the pH maintained at 7.5 for 1.5 hours. Cell walls were then washed three times by centrifugation and resuspension in water. The suspension was adjusted to pH 5.6 with dilute HCl followed by three water washes and finally suspended in water to a total volume of 220 ml. Deesterified cell walls at pH 4.4 were similarly prepared.

The composition of untreated and deesterified cell wall preparations, both suspensions at pH 4.4, are given in table I.

Preparing Tobacco Pith Homogenate: A 245 mm section of the stem above the fourth node of a 3.5 ft plant was used for this purpose. The pith was removed and cut into 3 mm sections. To the sections was added 7 ml of 0.1 M phosphate buffer (pH 7.0) and the mixture was ground in an Omnimixer[6] at 0° C.

Removal of Pectic Material from Cell Walls with Purified Polygalacturonase: For the specific removal of pectic material from Avena cell walls, to 50 ml of deesterified cell wall preparation (0.55 g dry wt of cell walls) were added 20 ml of 1 M acetate buffer at pH 5.0, 20 ml of 0.5 % ethylenediaminetetraacetate, 10 ml of H_2O, and 20 mg of highly purified polygalacturonase (0.40 polygalacturonase unit) (14). The reaction mixture was stirred slowly for 3 hours at 25° C, toluene was added as a preservative, and the mixture allowed to incubate for 6 days. Analysis of the supernatant by the method of McComb and McCready (16) showed that approximately 90 % of the cell wall pectic material had been extracted. This result was confirmed by analysis of the cell walls [by the method of McCready and McComb (17)] after adjusting the pH of the reaction mixture to 4.4, centrifuging, and washing the cell walls five times with water. Volume of the final suspension in water was 50 ml. Supernatant and washings from the polygalacturonase treatment

Table I

Composition of Avena Coleoptile Cell Wall Preparations

	Untreated	Deesterified
Dry wt, %[1]	0.81	0.74
Pectic material %[2]	5.1	5.5
CH_3OH, %[3]	0.40	0.03
Molar ratio CH_3OH/AUA	0.43	0.03
PE units/g[4]	0.11	0

[1] Dry weight of the aqueous suspension of cell wall preparation.

[2] Anhydrouronic acid on dry weight basis.

[3] For CH_3OH determinations the cell wall preparations were centrifuged, washed several times with acetone by suspension and recentrifugation, and then dried in vacuo. Results are reported on a dry weight basis.

[4] On dry weight basis.

[6] Mention of trade names, products or manufacturers does not imply endorsement by the U. S. Department of Agriculture over others not mentioned.

were combined and concentrated in vacuum. Through the use of ion-exchange resins and paper chromatography the only monosaccharides which were liberated from the cell walls by polygalacturonase were glucose and galacturonic acid.

The preparation of commercial pectinase treated cell walls, which resulted in the removal of other polysaccharides in addition to the pectic material, has been reported earlier (12).

Results

PE-binding Capacity of Cell Walls: The PE-binding capacities of untreated and deesterified cell walls were determined with partially purified PE preparation (fig 2). Assays of the supernatants showed that 80 % of the PE added, up to a concentration of 0.02 PE unit per mg of cell wall, was adsorbed by the cell walls. Untreated and deesterified cell walls behaved identically. The maximum amount of PE bound was 0.022 PE unit per mg of cell wall, an amount 200 times greater than the native PE content of cell walls. The adsorbed PE was firmly bound since repeated water washing failed to elute any of the enzyme. The bound PE could be completely eluted at pH 7.5 in the presence of 0.15 *N* NaCl. For example, when 0.01 PE unit of enzyme was added per mg cell wall, assays of the supernatant showed that 0.008 PE unit was bound. The cell walls were centrifuged and the pellet washed five times by suspension in water and centrifugation. The cell walls were then suspended in 0.15 *N* NaCl (7.4 mg of cell walls/10 ml of solution), the pH adjusted to 7.5 and after 15 minutes again centrifuged. Assays of the supernatant showed that 0.0082 PE unit had been eluted. Thus all of the bound PE was recovered by elution. Similarly, when cell walls which had been treated with 0.02 PE unit per mg and which had bound 0.016 unit per mg were water washed and extracted as above, 0.016 PE unit was eluted. Therefore, water washing failed to remove PE but the enzyme was completely eluted at pH 7.5 in the presence of 0.15 *N* NaCl. These results suggest that the binding of PE to cell walls is simply an ionic binding.

Absence of Effect by IAA on PE-binding Capacity of Cell Walls or PE Activity: Since it had been reported that IAA promotes the binding of PE to cell wall homogenates (7, 8, 9), the effect of IAA on the PE binding capacity of Avena coleoptile cell walls has been determined. The results are presented in table II. It is clear that IAA is without effect on the PE-binding capacity of cell walls. The amount of PE bound in the presence and absence of IAA was determined by elution of the enzyme from the water washed cell walls subsequent to binding. PE assays of the supernatants from the original adsorption mixture after two hours at 0° C agreed with the elution data. Since the PE-binding capacity of cell walls is large and since the binding is apparently ionic, it is not surprising that it is not influenced by IAA.

Table II

PE Bound to Cell Walls[1] in Presence and Absence of IAA[2]

IAA	PE Units added/mg cell wall × 100[3]	PE Units bound[4]/mg cell wall × 100
Absent	2.5	1.8
Present	2.5	1.8

[1] Deesterified preparation of Avena coleoptile cell walls at pH 4.4 was used.
[2] The concentration of IAA in the adsorption mixture was 1×10^{-4} M.
[3] On dry weight basis.
[4] As determined by elution of bound PE.

The effect of IAA on PE activity was re-examined using tobacco pith homogenates as the enzyme source. The homogenate contained 0.0042 PE unit per ml. When the homogenate was assayed in the presence of 1×10^{-4} M IAA (added to the substrate prior to adding the homogenate), an identical activity of 0.0042 PE unit per ml was recovered. The homogenate possessed an apparent PE content of 0.0031 unit per ml when the substrate was 0.06 M with respect to $CaCl_2$ rather than the usual 0.15 *N* with respect to NaCl. In the presence of 1×10^{-4} M IAA, an activity of 0.0030 PE unit per ml was found. Therefore, under the conditions investigated, IAA was without a detectable effect on PE activity. These results are contrary to those reported by Bryan and Newcomb (6). The reason for the difference is not apparent.

Binding Capacity of Cell Walls for Pepsin, α-Chymotrypsin, and Peroxidase: Avena coleoptile cell walls, both untreated and deesterified preparations, are capable of firmly binding large amounts of pepsin, α-chymotrypsin, and peroxidase. The binding of these enzymes to cell walls is illustrated in figure 3. With α-chymotrypsin the maximum amount bound was 0.85 mg of enzyme per mg (dry wt) of deesterified cell wall preparation. At concentrations of 0.6 mg per mg of cell walls or less, essentially all of the α-chymotrypsin was bound. The maximum binding capacity of esterified cell wall preparation was approximately 80 % that of the deesterified preparation. Like PE, α-chymotrypsin was firmly bound and remained on the cell walls after washing with water but could be eluted with 0.15 *N* NaCl at pH 7.5.

Even larger amounts of pepsin are bound to cell walls. At the lower levels of pepsin concentration, essentially all of the added enzyme was bound. Not shown in figure 3 is the fact that when 2.27 mg of pepsin was added per mg cell walls, 2.02 mg (89 %) was bound. The amount of pepsin bound of necessity was determined by assay of the supernatant because of the extreme instability of pepsin at pH 7.5 (20). Water washing of cell walls failed to remove any of the bound pepsin. Cell walls, which had bound 1.27 mg of pepsin and had subsequently been washed with

water were treated with 0.15 *N* NaCl solution at pH 6.0 [a pH at which pepsin is reasonably stable (20)]. This resulted in the elution of 58 % of the bound pepsin.

That the binding of enzymes to Avena coleoptile cell walls is apparently not a function of the isoelectric point of the enzyme is suggested by the data for pepsin and α-chymotrypsin. The isoelectric point of the former is pH 2.7 (20) and that of the latter pH 8.6 (2).

Peroxidase was also firmly bound to cell walls but the binding capacity was less than for proteolytic enzymes. The results in figure 3 were obtained by elution of the peroxidase from water-washed, peroxidase-treated cell walls. The elution procedure was the same as that used for PE. With peroxidase, the

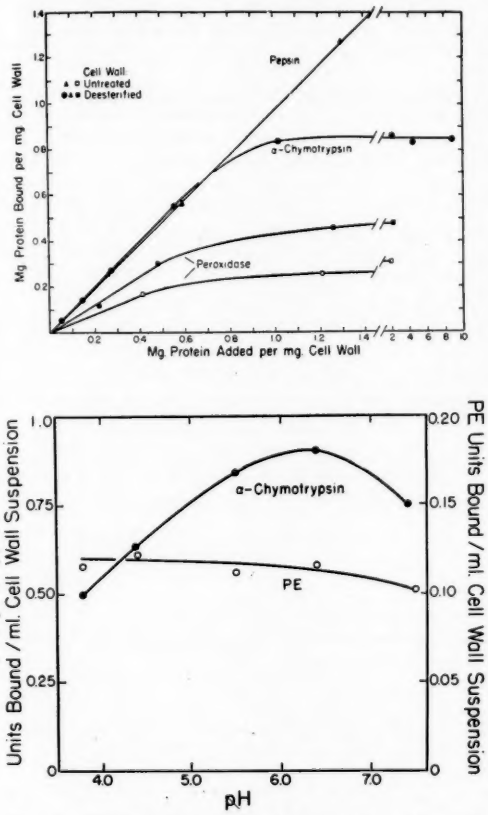


FIG. 3. Binding of pepsin, α-chymotrypsin and peroxidase to cell walls: Weighed samples of enzyme were dissolved in 1 ml of cell wall suspensions, both untreated and deesterified, and incubated for 2 hours at 0° C after which the suspensions were centrifuged and enzyme binding determined (see text). For binding α-chymotrypsin, cell wall suspensions at pH 5.6 were used. For the pepsin and peroxidase binding, suspensions at pH 4.4 were used.

FIG. 4. Effect of pH of cell walls on enzyme binding. (see text).

binding capacity of the cell walls is markedly increased by deesterification of their pectic substances. The maximum binding capacity of the untreated cell walls was 0.31 mg peroxidase per mg cell walls while that of the deesterified cell walls was 0.48 mg per mg. The ratio of binding capacity of deesterified/untreated cell walls, 1.55, is in reasonable agreement with the ratio of free carboxyl groups of the pectic substances of the two types of cell walls, 97 %/57 % or 1.67 (table I.). These results suggest that peroxidase may be bound to the free carboxyl groups of the pectic substances of the cell walls. Since larger amounts of the proteolytic enzymes are bound to cell walls, other binding sites must also be involved. This will be shown below to be the case.

Heating of cell walls had no effect on their PE binding capacity. To a deesterified cell wall preparation at pH 4.4 which had been heated in boiling water for five minutes and then cooled, was added 0.02 PE unit per mg cell walls. After incubating, centrifugating, water washing, and eluting the cell walls, 0.015 PE unit per mg was found to have been bound. This value is in good agreement with the comparable one of figure 2 (0.016). Similar results were obtained for binding α-chymotrypsin by heated cell walls.

Ethylenediaminetetraacetate at pH 6.0 neither altered the binding capacity of cell walls nor dissociated bound enzyme. Deesterified cell walls, pretreated with 1.8 mg of α-chymotrypsin per mg cell walls, was extracted with 0.1 % ethylenediaminetetraacetate solution at pH 6.0 for 1 hour. The supernatant contained only 6 % of the bound enzyme. Untreated cell walls at pH 4.4 (2 ml of suspension) were stirred with 10 ml of 0.5 % ethylenediaminetetraacetate at pH 6 for 3 hours. To these cell walls, after washing with 0.15 M acetate buffer at pH 4.4 and with water, was added 1 mg of α-chymotrypsin per mg of cell walls. The amount of enzyme bound as determined by the amount eluted was 0.64 mg per mg cell walls. This amount was the same as that of untreated cell walls at this pH (see fig 4). Therefore calcium or similar cations are not involved in binding enzymes to cell walls.

Living Avena coleoptile sections themselves do not bind PE. Sections (12 mm long) were prepared by the method used previously (12). To 0.5 ml of H_2O solution containing 0.16 PE unit was added 270 mg of sections and the mixture incubated for 2 hours at 0° C. PE assays of the solution showed that it still contained 0.16 PE unit. These results suggest that the cells must be ruptured in order to expose the PE binding sites of the cell walls.

EFFECT OF PH OF CELL WALLS ON ENZYME BINDING: To determine the effect of the pH of cell walls on their enzyme binding, 2.5 ml aliquots of water-washed deesterified cell walls at pH 4.4 were suspended in 10 ml of 0.15 M acetate buffers of pH 3.8, 4.4, 5.5, 6.4, and 7.5. The cell walls were centrifuged and washed five times with water. Each sample was then suspended in water to give the original volume of 2.5

ml. For determining the effect of pH on the binding of α-chymotrypsin, to 1 ml suspension of each was added 0.425 α-chymotrypsin unit (8 mg enzyme/ml or 1.08 mg/mg of cell walls) and for the effect on PE binding, 0.16 PE unit per ml (0.0216 PE unit per mg). After 1 hour at 0° C the cell walls were centrifuged and the supernatants assayed for α-chymotrypsin or PE. Each was then water washed and eluted as above. The results reported (fig 4) were those obtained by assays of the eluted enzymes which agreed well with those obtained by assays of the supernatants. The pH of the cell walls had little effect on PE binding (fig 4), but the pH of the cell walls markedly affected α-chymotrypsin binding with maximal binding occurring at pH 6.4 and minimal at pH 3.8. The difference observed in the effect of pH on the binding of these two enzymes suggests that they are bound to different sites on the cell walls.

DIFFERENT SITES FOR BINDING PROTEINS TO CELL WALLS: That there is more than one type of protein binding site on Avena coleoptile cell walls was established in several ways. The results (table III) show that the saturation of cell walls with α-chymotrypsin does not change the binding capacity of the preparation for PE and vice versa. Adding the second enzyme does displace (or exchange with) 20 to 25 % of the first enzyme used for saturation of the cell walls. The results clearly demonstrate that these two enzymes are principally bound to separate sites on cell walls. The limited displacement of the first enzyme by the second may possibly represent a third common site.

Further evidence of multiplicity of binding sites was obtained with the use of diisopropyl fluorophosphate inhibited α-chymotrypsin. The inhibited enzyme was added to deesterified cell walls at a concentration of 1.1 mg per mg cell walls and incubated for 2 hours at 0° C after which the cell walls were water washed and eluted as with those treated with the active enzyme. The results (table IV) show that the inhibited enzyme was firmly bound to cell walls in an amount 73 % that of the active enzyme. Cell walls saturated with the inhibited enzyme still bind 62 % of the amount of active enzyme bound by cell walls not so treated. Apparently, the active and inhibited α-chymotrypsin are largely bound to different sites.

Previous results demonstrated that binding PE and α-chymotrypsin to orange cell walls involved the pectic material of these cell walls since the specific removal of pectic substances therefrom with highly purified polygalacturonase (14) resulted in a proportional loss in the binding capacity for both enzymes (11). This was not the case, however, with Avena coleoptile cell walls where specifically removing 90 % of the pectic material of the cell walls decreased their binding capacity for PE and α-chymotrypsin by only

TABLE IV

BINDING OF α-CHYMOTRYPSIN AND ITS DIISOPROPYL FLUOROPHOSPHATE INHIBITION PRODUCT TO CELL WALLS[1]

CELL WALL TREATMENT	MG PROTEIN BOUND/MG CELL WALL[2]	
	α-CHYMOTRYPSIN	INHIBITED CHYMOTRYPSIN
None	0.56[3]	...
None	...	0.41[4]
Saturated[5] with inhibited chymotrypsin	0.35[3]	...

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] On dry weight basis.
[3] As determined by elution of the bound enzyme.
[4] As determined by N analysis of eluted protein.
[5] Cell walls were washed four times with H_2O by suspension and centrifugation after adsorption of the inhibited enzyme.

TABLE III

BINDING OF PE AND α-CHYMOTRYPSIN TO DIFFERENT SITES ON CELL WALLS[1]

CELL WALL TREATMENT	UNITS OF ENZYME/MG CELL WALL × 100[2]					
	ADDED		EXCHANGED[3]		BOUND[4]	
	PE	α-CHYMOTRYPSIN	PE	α-CHYMOTRYPSIN	PE	α-CHYMOTRYPSIN
None	2.2	...	...	...	1.3	...
None	...	5.7[6]	...	...	...	2.9
PE-saturated cell walls[5]	...	5.7[6]	0.23	...	1.1	3.0
α-Chymotrypsin saturated cell walls[5]	2.2	...	...	0.74	1.2	2.2

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] On dry weight basis.
[3] As determined by assay of supernatant after adding second enzyme.
[4] As determined by eluting the bound enzymes.
[5] Cell walls were washed four times with H_2O by suspension and centrifugation after the adsorption of first enzyme.
[6] 1.08 mg α-chymotrypsin per mg cell wall.

TABLE V

EFFECT OF POLYGALACTURONASE AND PECTINASE ON BINDING CAPACITY OF CELL WALLS[1]

CELL WALL TREATMENT	PROTEIN BOUND, %[2]		
	PE	α-CHYMOTRYPSIN	INHIBITED CHYMOTRYPSIN
None	100	100	100
PG	77	70	...
Pectinase	17	13	35

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] As determined by elution of the bound proteins.

23 and 30 %, respectively (table V). Accordingly, the pectic material of the cell walls plays only a minor part in binding these two enzymes. Removing all the pectic material together with other polysaccharides by commercial pectinase decreased the binding capacity of the cell walls for PE and α-chymotrypsin to approximately 15 % of the original value. On the other hand, 35 % of the binding capacity for inhibited chymotrypsin remained in cell walls so treated. These results clearly indicate the existence of more than one type of protein binding site in Avena cell walls.

EFFECT OF INCUBATING AVENA COLEOPTILE SECTIONS AT PH 4.4 AND PH 7.4 ON PE ACTION IN SITU: Auxin-induced elongation of Avena coleoptile sections proceeds at maximal rate at pH 4.4 and much more slowly at pH 7.4 (4). If the effect of auxin were mediated through binding of PE, then this binding should be maximal at pH 4.4 and PE action in situ should be maximal at pH 7.4. In order to obtain additional evidence on the auxin-PE binding hypothesis, sections were incubated in solutions at these two pH values. Avena coleoptile sections were prepared by the method previously used (12). One 20 g lot was added to 1 liter of 0.75 M phosphate buffer at pH 7.4 and another to 1 liter of 0.15 *N* acetate buffer at pH 4.4. Both were incubated for 18 hours with occasional stirring. After water washing, each was homogenized at 0° C with 80 ml of 0.15 *N* acetate buffer at pH 4.4. Assays of the homogenates showed that the sections incubated at pH 4.4 contained 0.0018 PE unit per g fresh weight and those incubated at pH 7.4, 0.0016 PE unit per g. The cell walls were centrifuged, water washed five times, and finally suspended in a total volume of 40 ml of water. The cell walls contained respectively 0.0014 and 0.0015 PE unit per g of original section. The cell walls were next filtered, washed with acetone, and then dried in a vacuum desiccator. The pectic substance content of each cell wall preparation was 5.1 % and the CH_3OH content was 0.42 % for cell walls prepared from sections incubated at pH 4.4; 0.36 % for sections incubated at pH 7.4. The pectic substance content was the same as that of cell walls prepared from non-incubated sections (table I). The lower CH_3OH content in cell walls from sections incubated at pH 7.4 indicates a slight in situ PE action during incubation at the higher pH value. The fact that the pH at which incubation was conducted does not influence the amount of PE accompanying the cell walls as well as the fact that pH 7.4 incubation resulted in only a trace of in situ PE action are both at variance with expectations of the auxin-PE binding hypothesis.

DISCUSSION

In the present investigations, no evidence has been obtained to support the suggestion that the effect of IAA in promoting the elongation of cells is mediated through an effect on PE. Avena cell walls are capable of firmly binding added PE in an amount approximately 200 times greater than that inherent to the cell walls. The binding is apparently ionic since all of the bound enzyme can be eluted with salt solutions at pH 7.5. IAA does not influence the binding of PE to cell walls nor does it affect the PE activity of tobacco pith homogenates. These results are not in accord with previous reports (6, 7, 8, 9, 23). Avena cell walls are also capable of firmly binding other proteins such as pepsin, peroxidase, α-chymotrypsin and its diisopropyl fluorophosphate inhibition product in large amounts. These proteins are likewise ionically bound. In other words, Avena coleoptile cell walls have much in common with certain chromatographic columns. On the other hand, living, intact Avena coleoptile sections do not bind added PE. Therefore the suggestion that IAA exerts its effect through a specific binding of PE is not tenable.

Previous results have shown that IAA does affect the pectic metabolism of the growing Avena coleoptile section (12). IAA increases the formation of the methyl ester of the hot-water-soluble portion of the pectic material of the cell wall without causing a detectable change in the degree of esterification of any of the pectic material of the cell walls. IAA causes an even greater increase in the rate of formation of methyl ester in the cold-water-soluble pectic material (12). Furthermore, IAA causes the same increase in the formation from glucose of the galacturonic acid residues of these two pectic fractions (1). This was demonstrated by incubating sections in the presence of C^{14}-labeled glucose with and without IAA, followed by isolation of the galacturonic acid and determination of its radioactivity. Whether or not the effect of IAA on the pectic metabolism of sections is its primary effect in promoting the elongation of cells remains to be proven. The fact that no IAA-induced net change in the degree of esterification of any of the pectic material of the cell walls could be detected constitutes additional evidence against the auxin-PE binding hypothesis.

SUMMARY

I. Aqueous suspensions of Avena coleoptile cell walls are capable of firmly binding added pectinesterase in amounts up to approximately 200 times that native to the cell walls. This is true for both native cell walls and walls in which the pectic methyl ester groups have been removed by pectinesterase. The

enzyme remained bound through exhaustive water washing but is readily eluted with 0.15 *N* NaCl at pH 7.5. The binding is apparently an ionic one.

II. The pectinesterase-binding capacity of cell walls is not influenced by added indoleacetic acid. The auxin is also without effect on the pectinesterase activity of tobacco pith homogenates.

III. Large amounts of pepsin, peroxidase, α-chymotrypsin and its diisopropyl fluorophosphate inhibition product are similarly firmly bound to Avena coleoptile cell walls. As much as 0.85 mg of α-chymotrypsin and 2.0 mg of pepsin are bound per mg of cell walls. These proteins are elutable by the method used for pectinesterase.

IV. Neither heating of cell walls in suspension nor treatment with ethylenediaminetetraacetate changes their binding capacity for pectinesterase or α-chymotrypsin. This sequestering agent also fails to liberate the bound enzymes.

V. Intact Avena coleoptile sections do not bind added pectinesterase.

VI. The pH of cell walls over the range of pH 3.8 to 7.5 does not influence their binding capacity for pectinesterase. The binding capacity for α-chymotrypsin is maximal at pH 6.4.

VII. Pectinesterase and α-chymotrypsin are bound to different sites in the cell wall since saturation with one does not influence the binding of the other. Saturation of cell walls with diisopropyl fluorophosphate-inhibited α-chymotrypsin decreases the binding capacity for α-chymotrypsin by 40 %.

VIII. The specific removal of pectic material from cell walls by highly purified polygalacturonase results in only partial reduction in the binding capacity for pectinesterase and α-chymotrypsin. Cell walls treated with pectinase, which removes polysaccharides in addition to pectic material, still retain approximately 15 % of their binding capacity for these two enzymes.

IX. Incubation of Avena coleoptile sections at pH 4.4 where auxin is most effective and at pH 7.4 where it is but slightly effective showed no difference in the amount of pectinesterase accompanying the cell walls or any significant difference in the degree of esterification of the pectic material in the cell walls.

X. No evidence has been obtained to support the view that the effect of auxin in promoting the elongation of cells is mediated through an effect on pectinesterase.

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Literature Cited

1. Albersheim, P. and J. Bonner. 1959. Metabolism and hormonal control of pectic substances. Jour. Biol. Chem. 234: 3105–3108.
2. Anderson, E. A. and R. A. Alberty. 1948. Homogeneity and the electrophoretic behavior of some proteins. II. Reversible spreading and steady-state boundary criteria. Jour. Phys. and Colloid Chem. 52: 1345–1364.
3. Balls, A. K. and E. F. Jansen. 1952. Stoichiometric inhibition of chymotrypsin. Advances in Enzymol. 13: 321–343.
4. Bonner, J. 1934. The relation of H ions to the growth rate of Avena coleoptiles. Protoplasma 21: 406–423.
5. Boos, R. N. 1948. Quantitative colorimetric determination of methanol with chromotropic acid reagent. Anal. Chem. 20: 964–965.
6. Bryan, W. H. and E. H. Newcomb. 1954. Stimulation of pectinesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum 7: 290–297.
7. Glasziou, K. T. 1957. The effect of 3-indolylacetic acid on the binding of pectin methylesterase to the cell walls of tobacco pith. Aust. Jour. Biol. Sci. 10: 337–341.
8. Glasziou, K. T. 1957. The effect of auxins on the binding of pectin methylesterase to cell wall preparations. Aust. Jour. Biol. Sci. 10: 426–434.
9. Glasziou, K. T. and S. D. Inglis. 1958. The effect of auxins on the binding of pectin methylesterase to cell walls. Aust. Jour. Biol. Sci. 11: 127–141.
10. Jansen, E. F., M-D. F. Nutting, R. Jang, and A. K. Balls. 1949. Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropyl fluorophosphate: Crystallization of inhibited chymotrypsin. Jour. Biol. Chem. 179: 189–199.
11. Jansen, E. F., R. Jang, and J. Bonner. 1960. Orange pectinesterase binding and activity. Food Res. 25: 64–72.
12. Jansen, E. F., R. Jang, P. Albersheim, and J. Bonner. 1960. Pectic metabolism of cell walls. Plant Physiol. 35: 87–97.
13. Kunitz, M. 1935. A method for determining the rennet activity of chymotrypsin. Jour. Gen. Physiol. 18: 459–466.
14. Lineweaver, H., R. Jang, and E. F. Jansen. 1949. Specificity and purification of polygalacturonase. Arch. Biochem. 20: 137–152.
15. MacDonnell, L. R., E. F. Jansen, and H. Lineweaver. 1945. The properties of orange pectinesterase. Arch. Biochem. 6: 389–401.
16. McComb, E. A. and R. M. McCready. 1952. Colorimetric determination of pectic substances. Anal. Chem. 24: 1630–1632.
17. McCready, R. M. and E. A. McComb. 1952. Extraction and determination of total pectic materials in fruits. Anal. Chem. 24: 1986–1988.
18. Morris, H. J., C. A. Weast, and H. Lineweaver. 1946. Seasonal variation in the enzyme content of eleven varieties of carrots. Bot. Gaz. 107: 362–371.
19. Northrop, J. H. 1946. Crystallization of pepsin from alcohol. Jour. Gen. Physiol. 30: 177–184.
20. Northrop, J. H., M. Kunitz, and R. M. Herriott. 1948. Crystalline enzymes. 2nd Ed. Columbia University Press, New York.
21. Ordin, L., R. Cleland, and J. Bonner. 1955. Influence of auxin on cell wall metabolism. Proc. Nat. Acad. Sci. 41: 1023–1029.
22. Sato, C. 1953. Methyl group synthesis in plant metabolism. Thesis. Michigan State University.
23. Yoda, S. 1958. Effect of auxin on pectic enzymes. Bot. Mag. Tokyo 71: 1–6.

EFFECTS OF TEMPERATURE AND HUMIDITY ON FOLIAR ABSORPTION AND TRANSLOCATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND BENZOIC ACID[1, 2, 3]

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Little is known concerning the effect of humidity on the absorption of foliarly applied materials or their subsequent translocation. It is generally believed that the higher the humidity at any one temperature, the larger the amount of a foliarly applied material that penetrates the leaf per unit time. Most of the evidence for such an effect has been obtained indirectly. Koontz and Biddulph (16) found that the amount of phosphorus translocated from a given compound seemed to be related to the drying time of the solution on the leaf. By adding glycerine to the treatment solution, the translocation of P^{32} from an application of KH_2PO_4 solution was increased. Other workers have reported increased penetration of foliarly applied materials by additives which increase moisture retention. The recent review by Currier and Dybing (7) covers this work. It has been suggested that thin aqueous films on the leaf surface are important in promoting the absorption of nutrient sprays, the existence of the film depends upon the vapor pressure gradient at the leaf surface (4).

In general, increasing temperature, within physiological limits, has been found to result in increased penetration. Rice (19) using red kidney bean plants studied the absorption of the ammonium salt of 2,4-D. He found that the amount absorbed was positively correlated with temperature over a three-level range of 46 to 58° F, 79 to 82° F and 86 to 92° F. Other workers using soybeans as the test plant found that with increasing temperature, there was an increase in the absorption of the sodium salt of 2,4-D (5,10). Barrier and Loomis (2) reported a temperature effect on the absorption of 2,4-D by soybean seedlings, but no effect upon the absorption of P^{32}. Increased rates of absorption with increasing temperature have been reported for Co^{60} (9) and manganese (18).

Known quantities of maleic hydrazide have been applied on several species of plants; the time course of absorption followed as it was affected by temperature and humidity (21). A variation in temperature at controlled humidities was found to have less effect on the absorption rate than a variation in humidity at controlled temperatures. Either an increase in temperature or humidity gave an increase in absorption of the maleic hydrazide.

The preponderance of evidence to date indicates a maximum in translocation over the range of 20 to 30° C. At temperatures below and above this range translocation is reduced (2, 12, 22).

MATERIALS AND METHODS

Selected seed of *Phaseolus vulgaris* L. var. Red Kidney was used. Seeds were germinated at 25° C in Vermiculite saturated with distilled water. All temperatures reported are accurate within ± 1° C and relative humidities are accurate to ± 3 % as measured by a hair hygrometer calibrated periodically with a hand phychrometer. Forty to 42 hours after sowing, 90 germinated seeds with radicles between 1.5 cm and 1 cm long were selected and planted in 4 inch pots containing Yolo clay loam soil fertilized with 16 ppm N and 6 ppm P on an oven dry weight basis. After subirrigation the pots were transferred to a growth chamber where they remained at 25° C until the 5th day after sowing, when the lights were turned on at 5:30 A.M. At this time the beans were in the crook stage. Subsequent growth conditions were: 15½ hours light, temperature 25° C, relative humidity 60 to 76 % and 8½ hours dark, temperature 15° C, relative humidity 85 to 95 %. Light was obtained from a fluorescent-incandescent source giving 1,100 to 1,300 ft-c at the level of the unifoliate leaves.

The beginning of the experimental temperature and humidity regimes coincided with initiation of the light period on the 9th day after planting the dry seeds. Generally, 60 selected plants were treated between 9:00 and 12:00 A.M. The first trifoliate leaves were beginning to unfurl from the bud at this time. Plants were watered before treating; in experiments at lower humidities, they also were watered several hours later. Treatment consisted of applying two 5 µl droplets on each side of a unifoliate leaf, approximately 2/3 cm from the major vein and 1½ cm from the base of the leaf. The treatment solution contained 10 µg acid equivalent of the triethanolamine salt of 2,4-dichlorophenoxyacetic acid or 5.52 µg acid equivalent of triethanolamine benzoate in water with 0.1 % Vatsol OT (sodium dioctyl sulfosuccinate). The C^{14} activity of carboxyl-labelled 2,4-D and benzoic acid was 6.03 mc/mM and 1.0 mc/mM, respectively. Ten replications of every treatment were harvested at 2, 4, 6, and, in two experiments, 8 hours after treatment. The harvest consisted of washing out the root systems and then simultaneously removing two 1 cm sections from the epicotyl by means of a three-bladed knife. The first cut was made as close

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to the junction of the petiole and epicotyl as possible. Remaining plant parts were quick-frozen between blocks of dry ice; when the harvest was completed they were lyophilized. The epicotyl sections were placed in small, numbered culture tubes; 0.5 ml 1 *N* NaOH was added to each, and the tubes were stoppered and placed in an oven at 60 to 65° C for 24 hours for digestion and extraction. A complete extraction of C^{14} was obtained. The extracts were plated, dried, and then counted in a gas flow counter. Due to the hygroscopicity of NaOH, planchets had to be stored at 55 to 60° C to give reproducible counts. The NaOH method is recommended when dealing with an easily subliming compound like benzoic acid since it prevented detectable loss of radioactivity.

To determine the chemical form in which the C^{14} was present at the time of extraction from epicotyls for counting, analyses were made of the fresh petioles of treated leaves of plants treated in experiment No. 2 at 30° C, high humidity since translocation was optimal under these conditions and any metabolic conversion would normally be highest at an elevated temperature. At the time of harvest of epicotyls the petioles were bulked, frozen in dry ice, and remained frozen until extraction. The extraction process consisted of grinding ten petioles in a small tissue grinder to which 3 ml of 0.1 *N* HCl was added. At the completion of grinding 0.5 ml of the unfiltered brei was added to a culture tube and 0.5 ml of 1 *N* NaOH added. Three 3 ml ether extractions were made of the remaining 2.5 ml of brei. After the ether extractions, 0.5 ml of the extracted brei was again added to a culture tube and 0.5 ml 1 *N* NaOH added. The NaOH solutions were placed in the oven at 60 to 65° C for 24 hours, cooled, plated, and counted in the same manner as epicotyl extracts. The ether extract of 2,4-D treated plants was spotted directly on Whatman No. 1 filter paper for chromatography. The ether extract of benzoate-treated plants was further extracted with 3 ml of 0.1 % Na_2CO_3 solution. This additional extraction was found necessary for benzoate-treated leaf petioles since in a preliminary experiment most C^{14} activity was lost upon evaporation of the ether extract. The Na_2CO_3 extract was spotted on Whatman No. 1 filter paper for chromatography. Controls consisted of untreated petioles to which stock radioactive 2,4-D or benzoic acid was added just prior to grinding. Controls were subjected to the same extraction and counting procedures as petioles from treated plants. The Andreae and Good (1) solvent system was used; it consisted of isopropyl alcohol, concentrated ammonium hydroxide, and water (80:10:10, v/v). A co-chromatogram of stock 2,4-D and benzoic acid was run.

Four representative 2,4-D and benzoate-treated, lyophilized plants of every harvest period were selected for radioautography. Since equivalent amounts of chemicals were applied but the activity differed by a factor of six, the benzoate-treated plants were autographed 6 weeks and the 2,4-D treated plants 1 week.

Only one environmental chamber was available; therefore, it was necessary to make successive tests at every selected temperature and humidity. To test the reproducibility of an experiment two tests were made at 25° C high humidity and two tests at 30° C high humidity. There was no significant difference for replications at the 5 % level. Experiments were conducted at 20, 25, 30, and 35° C at high and low humidities (table I).

Results and Discussion

It is evident that with an increase in temperature there was an increase in the amount of 2,4-D translocated to the epicotyl (figs 1 and 2). This could have resulted from any or all of three effects: A. Increased absorption, B. Increased translocation, C. A preferential accumulation of the 2,4-D in the epicotyl. The last explanation is the most unlikely of the three, for with increasing temperature, radioautograms showed no lack of activity in other parts of the plant. Rather, with an increase of temperature there was also an increase in activity in the petiole of the treated leaf and the hypocotyl. On the basis of the above, an increase in translocation would most likely account for the increase in activity. However, the increase in translocation probably results from a more rapid rate of absorption under conditions of high humidity. Figure 2 shows that at any given temperature for any given time, there was always less 2,4-D translocated into the epicotyl under a low humidity. In figure 1 it can be seen that the amount of 2,4-D translocated into the epicotyl per unit of time falls off between 4 and 6 hours at both 30 and 35° C. That the translocation of 2,4-D has stopped is not to be regarded as the only possible explanation of the lack of a further increase in activity in the period between 6 to 8 hours at 35° C. Another possible explanation is that the accumulation of 2,4-D by the epicotyl has reached an equilibrium where outflow becomes equal to inflow. The amount of 2,4-D translocated per unit of time at 20 and 25° C appears to be very much the same; however, at these temperatures evidence is lacking of a decrease in translocation between 4 and 6 hours or even between 6 and 8 hours at 25° C. No radioautograms were made of the 8 hour harvest period, so further clarification of this point is not

Table I

Temperature, Humidity, and Vapor Pressure Deficit of Experiments

Temp.	Experiment No.	Relative humidity	Av VPD MM Hg
20° C	1	73 % ± 5 %	4.74
20° C	1	48 % ± 4 %	9.13
25° C	1	70 % ± 5 %	7.13
25° C	2	70 % ± 5 %	7.13
25° C	1	34 % ± 5 %	15.70
30° C	1	70 % ± 5 %	9.56
30° C	2	70 % ± 5 %	9.56
30° C	1	40 % ± 3 %	19.12
35° C	1	74 % ± 5 %	10.98
35° C	2	70 % ± 5 %	12.70
35° C	1	38 % ± 4 %	26.18

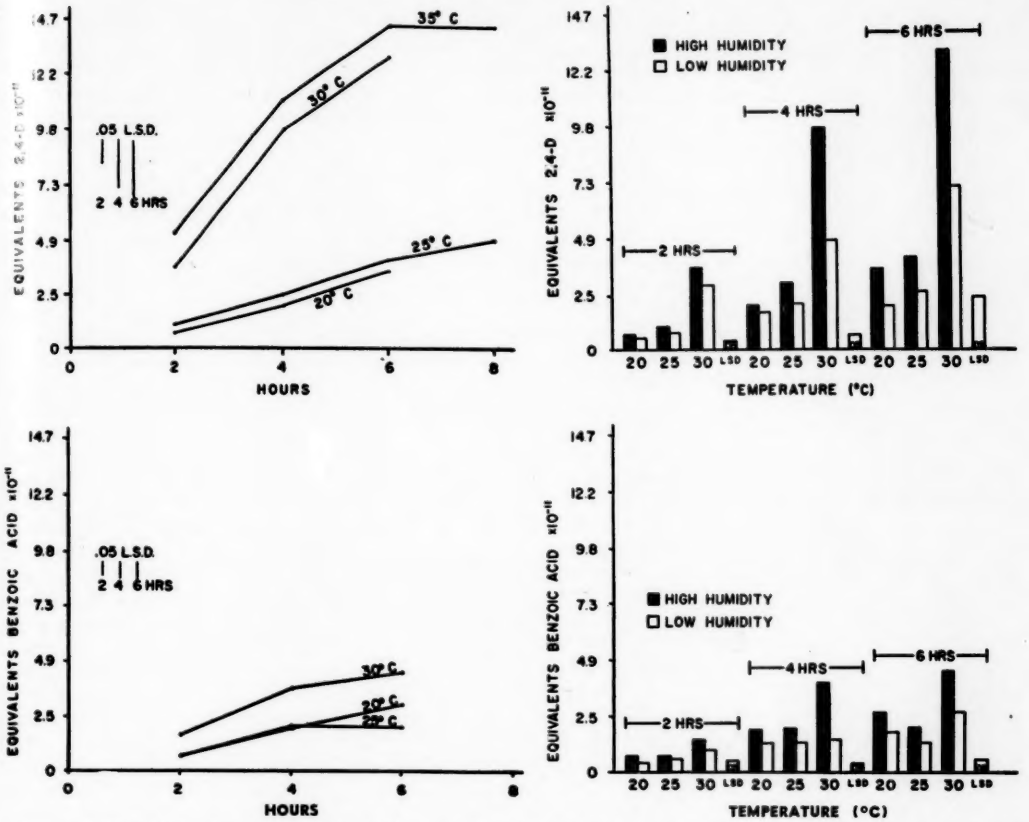

FIG. 1 (*upper left*). The relationship of the amount of 2,4-D absorbed and translocated to the epicotyl at indicated temperatures (high humidity) with increasing time.

FIG. 2 (*upper right*). The relationship of the amount of 2,4-D absorbed and translocated to the epicotyl at high and low humidities at indicated temperatures and time.

FIG. 3 (*lower left*). The relationship of the amount of benzoic acid (or a metabolic derivative) absorbed and translocated to the epicotyl at indicated temperatures (high humidity) with increasing time.

FIG. 4 (*lower right*). The relationship of the amount of benzoic acid (or a metabolic derivative) absorbed and translocated to the epicotyl at high and low humidities at indicated temperatures and time.

possible. Day (8), Rice (19), and Weaver and DeRose (24) found that under greenhouse conditions the translocation of 2,4-D into the stem was essentially completed 4.5, 4, and 5 hours, respectively, after treatment.

Figure 3 indicates that translocated activity in benzoate-treated plants did not vary between 20 and 25° C, but a marked increase was noted at 30° C. At any given temperature and time, the amount of benzoic acid translocated into the epicotyl is less at a low humidity than at a high humidity (fig 4).

Leaves of plants treated at 35° C (experiment No. 1) showed necrosis at the point of application, especially under the more humid conditions. The effect was more pronounced in 2,4-D treated plants. At this temperature high coefficients of variation were found; activities of epicotyl extracts were less than 30° C experiments. A rerun at the higher humidity did give the expected trend for 2,4-D (fig 1); but treated areas of leaves still showed considerable necrosis resulting from a surfactant effect.

No recovery data for 2,4-D are shown in table II as 95% of the C^{14} was found to be ether extractable after a six hour treatment period. Chromatograms indicated the bulk of the labelled ether-soluble material was 2,4-D. This finding is in accord with the literature (11, 13, 14, 15, 25).

Free benzoic acid is reported in some plants (3); however, it is rapidly metabolized in incubated pea epicotyl sections (1). The initial translocation of the C^{14} in benzoate-treated bean plants occurred primarily as benzoic acid or a dissociable salt thereof. With increasing time from treatment, it is apparently metabolized into other compounds possibly both before and after translocation. This deduction is made on the basis of ether solubility of the radioactive compounds

TABLE II

% ETHER-SOLUBLE RADIOACTIVE COMPOUNDS IN PETIOLES OF LEAVES TREATED WITH TRIETHANOLAMINE SALT OF BENZOIC ACID

TREATMENT TIME	EXTRACTION	CPM/10 PETIOLES	% ETHER SOLUBLE
2 hrs	Brei before extraction	580	78.1 %
	Ether extracted brei	127	
4 hrs	Brei before extraction	1,035	76.8 %
	Ether extracted brei	240	
6 hrs	Brei before extraction	2,220	67.2 %
	Ether extracted brei	728	
Control	Brei before extraction	6,200	96.5 %
	Ether extracted brei	218	

from petiole extracts (table II) and the identification of the translocate from chromatograms.

There is an apparent difference in mobility of the two compounds when applied to the bean leaf. The amount of benzoic acid translocated to the epicotyl at any given time, temperature, and humidity was

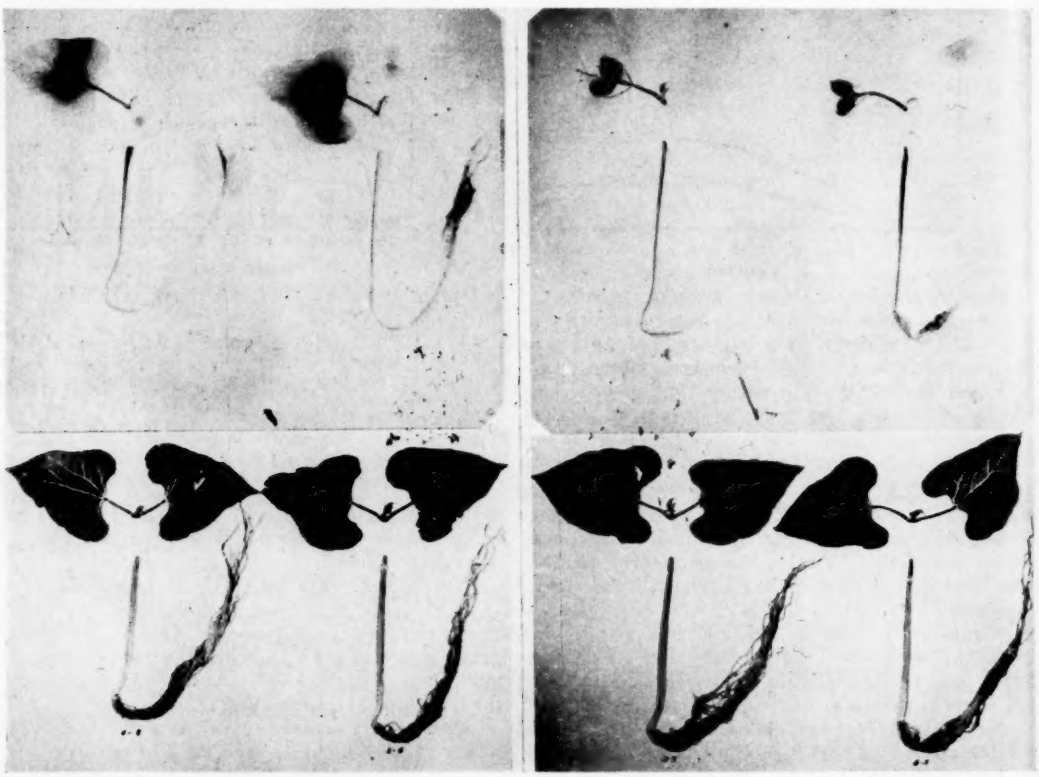

FIG. 5 (*left*). Distribution of benzoic acid or a metabolic derivative in bean plants treated at 30° C, low humidity for 4 hours. Upper section is radioautogram of treated plants shown in lower section. Missing epicotyl of plants was used in C^{14} analysis. The intensely dark area of roots on the radioautogram is associated with the adherence of soil particles.

FIG. 6 (*right*). Distribution of 2,4-D in bean plants treated at 30° C, low humidity for 4 hours. Upper section is radioautogram of treated plants shown in lower section.

always less than for a comparable 2,4-D treatment (compare fig 2 with fig 4). The more diffuse movement of benzoic acid in the treated leaf (fig 5) is in contrast to the polarized movement of 2,4-D (fig 6); the indication is that benzoic acid moves not only in the assimilate stream but spreads throughout the treated leaf. The diffuse spread of activity in the benzoate-treated leaf could be a movement via the symplast and/or apoplast (7) of either benzoic acid and/or a metabolic derivative. This movement is similar to the movement of the more water-soluble herbicides (6). Benzoic acid is much more soluble in water than 2,4-D.

A highly significant increase in epicotyl activity of 2,4-D treated plants was found upon an increase of temperature from 25 to 30° C at a high humidity (figs 1 and 2). A discrepancy in the results might be suggested, had not reruns been made at both temperatures. When a comparison is made of the activity in the epicotyl of plants treated with 2,4-D at temperatures of 20° C (high humidity) with those similarly treated at 30° C (high humidity) a Q_{10} of about four is apparent for the 4 hour treatment period. This is too high to be accounted for by an enzymatic reaction let alone a diffusional one. In the case of benzoic acid, a Q_{10} of about two is apparent from a comparison of the 20° C experiment with the 30° C experiment at a 4 hour harvest period (fig 3). The difference between the two Q_{10}'s may be accounted for by the loss of activity due to diffused movement in benzoate-treated plants. At even short periods of harvest there was a large difference between the activity in the epicotyl of plants treated at a high or a low humidity (figs 3 and 4). In general, little difference was noted in the speed of drying of applied droplets under the various humidities and temperatures, at least there was not enough difference to explain the results.

It was noted in several experiments that stomata were more frequently closed and their apertures smaller at lower humidities than at higher humidities. Plants were grown as previously described to study the degree of stomatal closure as it affected the uptake and translocation of 2,4-D. The experiment was run at 30° C, relative humidity 30 % ± 3 % and the plants were harvested 4 hours after treatment. Stomata of the plants were observed in situ under a compound microscope before treatment. Treatment solutions were applied as two 5 µl drops to areas previously described. Treatments consisted of:

I. 10 µg 2,4-D triethanolamine and 0.1 % Vatsol OT in 10 µl of distilled water.

II. 10 µg 2,4-D triethanolamine, 0.1 % Vatsol OT and 0.1 % glycerine as a humectant.

III. 10 µg 2,4-D triethanolamine and 0.1 % Vatsol OT; 10 minutes after application to the leaf the areas of treatment were rewet with 5 µl of water.

IV. One unifoliate leaf was bagged with a small polyethylene bag at the initiation of the light period. During and after treatment of the bagged leaf with 10 µg 2,4-D triethanolamine and 0.1 % Vatsol OT the bag was opened and remained open.

Adding a hygroscopic agent or rewetting of the treated area appears to be better than applying straight droplets at a low humidity (although not statistically significant at the tested levels) (table III). A rather large increase in absorption and translocation of 2,4-D is evident when the stomata were open at the time of treatment. That the droplet remained moist for a longer time, thus increasing uptake, was ruled out since the leaf was subjected to the low humidity upon treating and thereafter. It is believed that the greater activity in the epicotyl at higher humidities is at least partially the result of an increase in stomatal penetration. It has been reported that stomata open under a high humidity (23). Wilson (26) found that a greater percentage of stomata are open in most species he studied at higher temperatures than at lower ones. If the stomata were open more at the higher temperatures with a high humidity, this would account for the difference in absorption and translocation at the higher temperatures as found.

The hypothesis that temperature and humidity control the amount translocated by their effect on stomatal opening is further strengthened by the results shown in the low humidity experiments (fig 2). At low humidities with stomata frequently closed, penetration would be mainly rate-limited by the cuticle, thus giving a Q_{10} of 1.5 or higher if cuticular diffusion was associated with some mass movement through stomata. At the 4 hour harvest period, when a comparison of the activity in the epicotyl at 20 and 30° C is made, a Q_{10} of about two is derived.

Even if stomatal penetration is important, ectodesmata that have been found directly below the cuticle

TABLE III

STOMATAL OPENING AS RELATED TO PENETRATION AND TRANSLOCATION

CHEMICAL APPLICATION	FURTHER TREATMENT	AV CPM* **	STOMATA OPEN	OPENING WIDTH
Triethanolamine salt of 2,4-D and 0.1 % Vatsol OT	None	141	25 %	1/3
"	Treated area rewet	195	25 %	1/3
"	0.1 % glycerin to treatment solution	209	25 %	1/3
"	Treated leaf bagged	449	75 %	3/4-full

* $LSD_{0.05} = 94$; $LSD_{0.01} = 126$

** cpm of 0.1 ml of 0.5 ml 1 *N* NaOH extract of bean epicotyls. To convert cpm to equivalents of 2,4-D per 2 cm section of epicotyl, multiply cpm by 2.5×10^{-13}.

(17) should be given due consideration (20). The direct involvement of these ectodesmata in the penetration of foliarly applied herbicides or nutrients has not as yet been shown; they may function more efficiently at higher humidities in absorption phenomenon.

Summary and Conclusions

Radioautograms were used as a qualitative estimate and counting as a quantitative estimate of the absorption and translocation of C^{14}-labelled 2,4-D and benzoic acid applied to red kidney bean plants for 2, 4, 6, and 8 hours. The compounds were applied as the triethanolamine salts.

With increasing temperatures from 20 to 30° C increased absorption and translocation of both compounds were found. At temperatures of 20, 25, and 30° C less 2,4-D or benzoic acid was absorbed and translocated at low humidities (34–48 %) than at high humidities (70–74 %). The increased absorption and translocation at a higher humidity was correlated with the degree of stomatal opening.

The movement of 2,4-D in the leaf, as indicated by radioautograms, was confined in general to the vascular bundles and followed the route of the assimilate stream out of the leaf and into the stem, bud, and roots. The benzoate-treated plants showed not only a similar movement out of the leaf but also a diffuse movement away from the treated areas to all parts of the treated leaf.

Studies involving extraction of the radioactivity and partitioning in solvents coupled with chromatography showed the bulk of the radioactivity was being translocated as free 2,4-D, and benzoic acid or dissociable salts thereof.

Acknowledgments

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Literature Cited

1. Andreae, W. A. and N. E. Good. 1957. Studies on 3-indoleacetic acid metabolism. IV. Conjugation with aspartic acid and ammonia as processes in the metabolism of carboxylic acids. Plant Physiol. 32: 566–572.
2. Barrier, G. E. and W. E. Loomis. 1957. Absorption and translocation of 2,4-dichlorophenoxyacetic acid and P^{32} by leaves. Plant Physiol. 32: 225–231.
3. Bonner, J. Plant Biochemistry. 1950. Pp. 162–163. Academic Press, Inc., New York.
4. Boynton, D. 1954. Nutrition by foliar application. Ann. Rev. Plant Physiol. 5: 31–54.
5. Bryan, A. M., D. W. Staniforth, and W. H. Loomis. 1950. Absorption of 2,4-D by leaves. No. Cent. Weed Control Conf. Proc. 7: 92–95.
6. Crafts, A. S. and S. Yamaguchi. 1958. Comparative tests on the uptake and distribution of labeled herbicides by *Zebrina pendula* and *Tradescantia fluminensis*. Hilgardia 27: 421–454.
7. Currier, H. B. and C. D. Dybing. 1959. Foliar penetration of herbicides—review and present status. Weeds 7: 195–213.
8. Day, B. E. 1952. Absorption and translocation of 2,4-dichlorophenoxyacetic acid by bean plants. Plant Physiol. 27: 143–152.
9. Gustafson, F. G. 1956. Absorption of Co^{60} by leaves of young plants and its translocation through the plant. Amer. Jour. Bot. 143: 157–160.
10. Hauser, E. W. 1955. Absorption of 2,4-dichlorophenoxyacetic acid by soybean and corn plants. Agron. Jour. 47: 32–36.
11. Hay, J. R. and K. V. Thimann. 1956. The fate of 2,4-dichlorophenoxyacetic acid in bean plant seedlings. I. Recovery of 2,4-dichlorophenoxyacetic acid and its breakdown in the plant. Plant Physiol. 31: 382–387.
12. Hay, J. R. and K. V. Thimann. 1956. The fate of 2,4-dichlorophenoxyacetic acid in bean seedlings. II. Translocation. Plant Physiol. 31: 446–451.
13. Holly, R. W. 1952. Studies of the fate of radioactive 2,4-dichlorophenoxyacetic acid in bean plants. II. A water soluble transformation product of 2,4-D. Arch. Biochem. Biophys. 35: 171–175.
14. Jaworski, E. G. and J. S. Butts. 1952. Studies in plant metabolism. II. The metabolism of C^{14}-labelled, 2,4-dichlorophenoxyacetic acid in bean plants. Arch. Biochem. Biophys. 38: 207–218.
15. Jaworski, E. G., S. C. Fang, and V. H. Freed. 1955. Studies in plant metabolism. V. The metabolism of radioactive 2,4-D in etiolated bean plants. Plant Physiol. 30: 272–275.
16. Koontz, H. and O. Biddulph. 1957. Factors affecting absorption and translocation of foliar applied phosphorus. Plant Physiol. 32: 463–470.
17. Lambertz, P. 1954. Untersuchungen über das Vorkommen von Plasmodesmen in den Epidermisaussenwänden. Planta 44: 147–190.
18. Mederski, H. J. and D. J. Hoff. 1958. Factors affecting absorption of foliar applied manganese by soybean plants. Agron. Jour. 50: 175–178.
19. Rice, E. L. 1948. Absorption and translocation of ammonium 2,4-dichlorophenoxyacetate by bean plants. Bot. Gaz. 109: 301–314.
20. Sievers, A. 1959. Untersuchungen über die Darstellbarkeit der Ectodesmen and ihre Beeinflussung durch physikalische Faktoren. Flora 147: 263–316.
21. Smith, A. E., J. W. Zukel, G. M. Stone, and J. A. Riddell. 1959. Factors affecting the performance of maleic hydrazide. Agr. and Food Chem. 7: 341–344.
22. Swanson, C. A. and J. B. Whitney. 1953. Studies on the translocation of foliar applied P^{32} and other radioisotopes in bean plants. Amer. Jour. Bot. 40: 816–823.
23. Volck, W. H. 1903. Spraying with distillates. Cal. Agr. Exp. Sta. Bull. 153: 1–31.
24. Weaver, R. J. and H. R. Derose. 1946. Absorption and translocation of 2,4-dichlorophenoxyacetic acid. Bot. Gaz. 107: 509–521.
25. Weintraub, R. L., J. W. Brown, M. Fields, and J. Rohan. 1952. Metabolism of 2,4-dichlorophenoxyacetic acid. I. $C^{14}O_2$ production by bean plants treated with labeled 2,4-dichlorophenoxyacetic acid. Plant Physiol. 27: 293–301.
26. Wilson, C. C. 1948. The effect of some environmental factors on the movements of guard cells. Plant Physiol. 23: 5–37.

EFFECT OF AUXIN UPON LOSS OF CALCIUM FROM CELL WALLS[1, 2]

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In 1955, Bennet-Clark (2) and Carlier and Buffel (4) independently suggested that auxin induces elongation of stem and coleoptile tissues by removing calcium from the cell wall. According to this calcium-bridge hypothesis, the factor which limits cellular expansion is the number of calcium ions which cross-link the pectin chains of the cell wall. Removing such cross-linkages by auxin would result in elongation. It has been suggested that auxin brings about this removal of calcium by chelation (2, 9, 19) or by methylation of the carboxyl groups to which the calcium is attached (12, 13).

Considerable evidence from work with stems and coleoptiles has been used to support such a theory. Calcium increases the rigidity of cell walls (1, 15) and inhibits elongation (8, 16, 19). Ethylenediamine tetraacetic acid (EDTA), which might be expected to remove calcium from the wall by chelation, promotes elongation (2, 9, 19). Auxin induces an increase in the transfer of methyl groups from methionine to the pectin of Avena (12, 13) and maize (6) coleoptile cell walls.

The fact that calcium promotes root elongation suggests that calcium bridges do not control the elongation of root cells (3).

If this theory is correct, adding auxin to a tissue should result in a loss of calcium from the cell walls. The loss of calcium from whole Avena coleoptile tissues has been examined by Thimann and Takahashi. Initial experiments suggested that the combination of auxin and EDTA enhanced this calcium loss (17) but subsequent experiments have indicated that the increase is not significant (Thimann, personal communic.)

This calcium-bridge hypothesis has now been directly tested. The effect of auxin on the loss of Ca^{45} from cell walls has now been determined in Avena coleoptile and maize mesocotyl tissue.

MATERIALS AND METHODS

Experiments were first performed with mesocotyl sections of *Zea mays* L. and were later repeated with coleoptile sections of *Avena sativa* L. var. Victory in order to compare the results of this study with the methylation studies of Ordin et al (12, 13). Labeling of the cell walls was initially obtained by pretreating sections with Ca^{45}. However, it was thought that the carboxyl-calcium bonds formed in this way might be different from those formed at the time of cell wall synthesis. If this were so, with sections treated with Ca^{45} only the new calcium-carboxyl bonds would involve Ca^{45} and the magnitude of the loss of Ca^{45} would not be a true indication of the amount of loss of total calcium from the walls. Therefore in some experiments Avena seedlings were grown from germination in a solution which contained Ca^{45} so as to be certain that the calcium present in the walls would be uniformly radioactive.

Maize mesocotyl sections were obtained as follows. Maize seeds were germinated in moist sand and allowed to grow in the dark for about 90 hours. Mesocotyls of 2.5 to 3.5 cm in length were then selected and one 5 mm section was excised from each mesocotyl, using a double-bladed cutter. The apical incision was 1 mm below the node. Avena coleoptile sections were obtained in the manner of McRae and Bonner (11).

The sections were incubated for 4 to 10 hours in 20 ml of water containing $Ca^{45}Cl_2$ in a concentration of either 1 or 2.2×10^{-6} M. $Ca^{45}Cl_2$ had a specific activity of 1.4 mC/mg. The sections were then collected, rinsed with distilled water, and placed in distilled water for 1 to 2 hours to remove excess Ca^{45}. One lot of sections was then harvested at this point in order to determine the initial labeling of the cell wall; the remaining sections were ready for the experimental treatment.

Alternatively, Avena seeds were soaked for 18 hours and husked. Four hundred seeds were then placed on dacron gauze mesh which was stretched over a frame and suspended so that the surface of the mesh barely touched the surface of 1 liter of 2×10^{-7} M $Ca^{45}Cl_2$ solution. After about 70 hours, coleoptiles of 2.5 to 3.0 cm in length were selected and a 10 mm section was excised from each coleoptile. The apical cut was 3 mm below the tip. The central leaf was removed from the Avena coleoptile sections in every experiment. Sections were washed for 30 minutes in water. One lot of sections was harvested in order to determine the initial labeling of the cell wall; the remaining sections were ready for use.

Sections whose cell walls were labeled with Ca^{45} were then incubated for 8 to 24 hours in 20 ml of 1 % sucrose solution, $\pm$ 5 ppm indoleacetic acid (IAA). In many experiments this solution also contained EDTA due to the report that EDTA facilitated the loss of calcium from Avena coleoptile tissues (17). EDTA was used in a concentration of 0.001 M and unbuffered since these conditions have been reported to enhance the growth of Avena coleoptile sections (3). At the end of the incubation, sections were col-

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TABLE I

EFFECT OF AUXIN ON LOSS OF Ca^{45} FROM MAIZE MESOCOTYL CELL WALLS IN PRESENCE (A) AND ABSENCE (B) OF EDTA

	INCUBATION MEDIUM		FR. WEIGHT	CPM Ca^{45}/10 MG CELL WALL
A	Water	Initial	...	2,570
		+ Auxin	...	2,240
		− Auxin	...	2,310
B	EDTA	Initial	410 mg	5,930
		+ Auxin	655 mg	4,780
		− Auxin	465 mg	4,300

Experimental conditions: A) 8 hours in 10^{-6} M $Ca^{45}Cl_2$ (1.7×10^5 cpm/20 ml). 1 hour wash in water. 8 hours in water, ± 5ppm IAA. B) 10 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/20 ml), + 1 % sucrose. 2 hour wash in water. 24 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. Walls prepared as explained in text. Ca^{45} extracted with hot 0.01 M EDTA.

lected, thoroughly rinsed with water, blotted dry with filter paper, and ground rapidly in a mortar with 2 ml of cold water. Cell wall debris was then separated from the water-soluble and protoplasmic materials by repeated washing and filtrating with cold water on a sintered glass funnel. Unless otherwise stated, the Ca^{45} was then removed from the wall by extracting the walls twice for 10 minutes with 2 ml of hot 0.01 M EDTA. After extraction almost no radioactivity remained in the wall fraction. The extracts were combined and an aliquot was plated on an aluminum planchet and counted with a thin-window Geiger-Muller tube. Fresh weights of the sections were determined before and after the final incubation in order to determine the effect of auxin upon expansion. Weight of the dry cell wall was determined after extraction upon air-dried residues. Each individual treatment contained sufficient material to give about ten milligrams weight of dry cell wall. All manipulations were carried out under a weak red light.

RESULTS

The effect of auxin on the loss of calcium from maize mesocotyl cell walls is shown in table I. In the initial experiments EDTA was added to the experimental solution because of the report of Thimann and Takahashi (17) that the loss of calcium from whole Avena coleoptile tissues was facilitated by EDTA. Carr and Ng (5) have reported that EDTA actually prevents the loss of calcium from wheat coleoptile walls. Therefore the experiments were repeated with the EDTA omitted. In both cases the results were the same: during the incubation period calcium is lost from the cell wall but auxin does not enhance this process. In most experiments less cell wall calcium was lost in auxin-treated tissues than in the controls. This difference is so small that it does not seem to be significant.

Calcium may be bound to carboxyl groups of either short-chain (pectin) or long-chain (protopectin) pectic substances. Auxin might simply be causing a redistribution of calcium between these two components rather than a loss from the walls. It has been shown that this does not occur by actually separating the calcium associated with the hot-water-soluble and hot-dilute-acid-soluble fractions of the wall. This has been done as follows. Sections after incubation with or without auxin were ground up and the wall fraction was separated in the usual manner. Calcium was then removed from the wall in three steps. Walls were extracted for 18 hours with 4 ml of cold 2:1 benzene-ethanol to remove any calcium associated with lipids. Then the walls were dried and the pectin and its associated calcium were removed by extracting the walls twice with 2 ml of hot water. Protopectin was removed by extracting twice with 2 ml of hot 0.01 M EDTA. No label remained after this extraction. It can be seen from the data of table II that auxin does not cause a redistribution of calcium between pectin and protopectin.

The effect of auxin on the loss of calcium from

TABLE II

FRACTIONATION OF MAIZE MESOCOTYL CELL WALL CALCIUM BY EXTRACTION

	ELONGATION	CPM Ca^{45}/10 MG CELL WALL, EXTRACTED BY:			TOTAL
		BENZ/ETOH	WATER	EDTA	
+ Auxin	22 %	7	1,070	1,930	3,007
− Auxin	4 %	15	995	1,770	2,780

Experimental conditions: 6 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/20 ml). 1 hour in water. 10 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. Walls prepared as explained in text.

Avena coleoptile cell walls is shown in table III. Regardless of whether the calcium had been introduced to sections or seedlings, auxin had no effect on the loss of calcium from the walls, although it was inducing a large growth response at the same time.

Note that the amount of calcium lost from the cell wall depends upon both the tissue and the method by which calcium was introduced to the tissue. Calcium is lost much more easily from Avena coleoptile section cell walls than from the walls of maize mesocotyl sections. Introduction of calcium into the tissue at the time of wall synthesis renders it less easily lost from the walls.

Discussion

The calcium-bridge hypothesis has been one of the most promising of the recent theories of the mechanism of auxin-induced elongation. Its attraction lies in the fact that it explains and correlates a number of hitherto unrelated facts: the inhibition of growth by calcium and its reversal by potassium ions, the promotion of elongation by chelating agents, and the increased methylation of pectin in the presence of auxin. The main point of this hypothesis is that auxin causes a removal of calcium ions from the cell wall and this loss of calcium ions then permits elongation to occur. This hypothesis has now been directly tested. Auxin does not cause an increased loss of calcium from cell walls, nor does it cause a redistribution of calcium between the short-chain and long-chain pectic substances of the wall. The calcium-bridge hypothesis appears to be incorrect.

There are three ways in which it might be argued that these results are inconclusive. First, it has not been directly shown that the calcium removed from the wall was actually bound to pectic substances. However Jansen et al (10) have shown that the calcium-binding capacity of the wall measured by exchange with Ca^{45} is identical with the cation exchange capacity calculated from the amount of carboxyl groups in the wall. This suggests that calcium bound in the cell wall must be attached to pectic materials.

If the calcium involved in auxin-sensitive bridges is only a small part of total wall calcium, an effect of auxin upon this fraction might be obscured by the rest of the calcium. With Avena seedlings grown in a $Ca^{45}Cl_2$ solution, even if it is assumed that only 10 % of cell wall calcium comes from the solution, not more than 1 % of the cell wall carboxyl groups is bound to calcium. In order for the effect of auxin upon the loss of calcium to be missed, the bridges broken by auxin must involve less than 0.1 % of cell wall carboxyl groups. Doubt is cast as to whether or not such a low concentration of calcium-bridges can be controlling growth by the finding of Cooil and Bonner (8) that the concentration of calcium needed to saturate the cation binding sites of the cell wall is only ten times as high as the concentration which causes the least detectable inhibition of growth. This suggests that 10 % of all wall carboxyl groups must be bound to calcium ions to result in a measurable change in the growth rate.

Finally, the effect of auxin upon calcium associated with cold-water-soluble pectin has not been examined. This type of pectin, whose metabolism is influenced by auxin (10), was discarded during the preparation of the cell walls. However it seems unlikely that any auxin-induced loss of calcium from this fraction can promote wall plasticity and elongation when the presence of the calcium does not even render this pectin insoluble.

The evidence would seem to show conclusively that the calcium-bridge hypothesis is incorrect in regard to Avena coleoptile and maize mesocotyl tissues. In view of this conclusion, it would be interesting to re-examine the evidence in favor of this theory.

Calcium ions inhibit auxin-induced elongation in the Avena coleoptile (8, 16). This is due, in part, to a stiffening of the cell wall (15). Monovalent ions such as potassium reverse this calcium-induced stiffening but have no effect on wall plasticity in Avena coleoptile tissues to which calcium has not been added (8, 15). Calcium must certainly cause this stiffening by formation of calcium bridges, but it has

Table III

Effect of Auxin on Loss of Ca^{45} From Avena Coleoptile Cell Walls as Related to Method of Introducing Ca^{45} Into Tissues. Wall Labeled by Introducing Ca^{45} to Sections (A) or Seedlings (B)

	Ca^{45} Introduced to		Growth	cpm Ca^{45}/10 mg cell wall
A	Sections	Initial	...	5,930
		+ Auxin	29 %	1,485
		− Auxin	15 %	1,580
B	Seedlings	Initial	...	5,540
		+ Auxin	42 %	3,300
		− Auxin	21 %	3,300

Experimental conditions: A) 4 hours in 2.2×10^{-6} M $Ca^{45}Cl_2$ (3.7×10^5 cpm/ 20 ml). 1 hour in water. 17 hours in 0.001 M EDTA, + 1 % sucrose, ± 5 ppm IAA. B) Seedlings grown in 2×10^{-7} M $Ca^{45}Cl_2$ (1.7×10^6 cpm/liter). Sections 24 hours in 0.001 M EDTA, + 0.0025 M K–Maleate (pH 4.8), + 1 % sucrose, ± 5 ppm IAA. Walls prepared and Ca^{45} extracted as explained in text.

yet to be conclusively shown that such cross-linkages occur in cell walls in the absence of added calcium, although it is probable that they exist in at least small amounts since the cell wall contains calcium. The fact that elongation is inhibited by calcium cannot be taken as evidence to indicate that auxin must remove calcium from the cell wall in order for elongation to occur.

Chelating agents promote shoot growth (2, 9, 19). It appears that these agents do not remove calcium from cell walls, however (5). IAA has been shown to form complexes with copper and iron, but all attempts to show IAA-calcium complexes have failed (7, 14). The effect of chelates and auxin upon growth does not seem to be due to any ability on their part to chelate cell wall calcium.

The auxin-induced transfer of methyl groups from methionine to hot-water-soluble pectin occurs in Avena coleoptiles (12, 13), but cannot be demonstrated in maize mesocotyls (6). Although this may only be a reflection of a marked preference by maize mesocotyls for methyl groups from some other source than methionine, the necessity of methylation of this pectin for auxin-action must be considered to be in doubt.

Thimann and Takahashi (17) reported that the loss of calcium from whole Avena coleoptile sections is enhanced by a combination of auxin and EDTA. They have since concluded that this effect is not large enough to be significant (Thimann, personal communic.) and that auxin does not cause a loss of calcium from the cell wall.

While the increase in cell wall rigidity upon adding calcium ions is almost certainly due to formation of calcium cross-linkages, there is no conclusive evidence that the rigidity of the walls in the absence of *added* calcium is actually due to calcium cross-linkages. But perhaps wall rigidity is due to other kinds of cross-linkages, such as ones involving sulfhydryl or amide bonds, and it is these linkages which are severed by auxin, leading to elongation.

Summary

The calcium-bridge hypothesis of auxin-action has been put to a direct test by determining the effect of auxin upon the loss of Ca^{45} from cell walls. The loss of calcium from Avena coleoptile and maize mesocotyl cell walls is not enhanced by auxin, irrespective of whether the Ca^{45} was introduced into the walls at the time of wall synthesis or only afterwards. Auxin does not cause a redistribution of calcium between pectin and protopectin. It would appear that the removal of calcium cross-linkages is not mediated by auxin and that the calcium-bridge hypothesis is incorrect.

Acknowledgments

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Literature Cited

1. Adamson, D. and H. Adamson. 1958. Auxin action on coleoptiles in the presence of nitrogen and at low temperature. Science 128: 532–533.
2. Bennet-Clark, T. A. 1955. A hypothesis on salt accumulation and the mode of action of auxin. In: The Chemistry and Mode of Action of Plant Growth Substances, R. L. Wain and F. Wightman, eds. Pp. 284–294. Buttersworth Scientific Publications, London.
3. Burström, H. 1952. Studies on growth and metabolism of roots. VIII. Calcium as a growth factor. Physiol. Plantarum 5: 391–402.
4. Carlier, A. and K. Buffel. 1955. Polysaccharide changes in the cell walls of water absorbing potato tuber tissue in relation to auxin action. Acta Bot. Néerl. 4: 551–564.
5. Carr, D. J. and E. K. Ng. 1959. The sequestration of calcium from preparations of wheat coleoptile cell walls. Physiol. Plantarum 12: 264–274.
6. Cleland, R. 1960. Auxin-induced methylation in maize. Nature 185: 44.
7. Cohen, D., B. I. Ginzburg, and C. Heitner-Wirguin. 1958. Metal chelating properties of plant growth substances. Nature 181: 686–687.
8. Cooil, B. and J. Bonner. 1957. Effects of calcium and potassium ions on the auxin-induced growth of Avena coleoptile sections. Planta 48: 696–723.
9. Heath, O. V. S. and J. E. Clark. 1956. Chelating agents as plant growth substances. Nature 177: 1118–1121.
10. Jansen, E. F., R. Jang, P. Albersheim, and J. Bonner. 1960. Pectic metabolism of growing cell walls. Plant Physiol. 35: 87–97.
11. McRae, H. and J. Bonner. 1953. Chemical structure and antiauxin activity. Physiol. Plantarum 6: 485–510.
12. Ordin, L., R. Cleland, and J. Bonner. 1955. Influence of auxin on cell-wall metabolism. Proc. Nat. Acad. Sci. 41: 1023–1029.
13. Ordin, L., R. Cleland, and J. Bonner. 1957. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32: 207–212.
14. Recaldin, D. A. and O. V. S. Heath. 1958. Chelation or complex-formation by indoleacetic acid in vitro. Nature 182: 539–540.
15. Tagawa, T. and J. Bonner. 1957. Mechanical properties of the Avena coleoptile as related to auxin and to ionic interactions. Plant Physiol. 32: 207–212.
16. Thimann, K. V. and C. L. Schneider. 1938. The role of salts, hydrogen-ion concentration and agar in the response of the Avena coleoptile to auxin. Amer. Jour. Bot. 25: 270–280.
17. Thimann, K. V. and N. Takahashi. 1958. The action of chelating agents on growth of Avena. Plant Physiol. 33 suppl.: xxxiii.
18. Thimann, K. V., R. R. Slater, and G. S. Christiansen. 1950. The metabolism of stem tissue during growth and its inhibition. IV. Growth inhibition without enzyme poisoning. Arch. Biochem. 28: 130–137.
19. Weinstein, L. H., A. M. Meiss, R. L. Uhler, and E. R. Purvis. 1956. Growth promoting effect of ethylenediamine tetraacetic acid. Nature 178: 1188.

ETHIONINE AND AUXIN-ACTION IN AVENA COLEOPTILE[1, 2]

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Ethionine has been shown by Schrank (12) to be an inhibitor of auxin-induced elongation in the Avena coleoptile. Since the inhibition can be reversed by methionine, it has been concluded that ethionine acts by affecting some facet of methionine metabolism. Work with animal tissues has shown that there are two ways in which this can occur: ethionine can antagonize the incorporation of methionine into proteins and thus block protein synthesis (14) or it can inhibit transmethylation (13). Recent work has suggested that ethionine may be inhibiting elongation in the Avena coleoptile by interfering with transmethylation. Auxin has been shown to cause an increase in the rate of transfer of methyl groups from methionine to cell wall pectin in this tissue (11). If this transmethylation is obligatory for auxin-action, inhibition of this methylation by ethionine would lead to an inhibition of elongation. This theory is only tenable if ethionine actually does inhibit cell wall methylation and this has not been previously demonstrated. It was the purpose of this investigation to see if such an inhibition could be found.

Schrank has shown that ethionine inhibits total elongation (12). Total elongation (TE) is the sum of reversible (ES) and irreversible elongation (IE). The inhibition of TE by ethionine has now been separated into inhibitions of ES and IE and the rapidity of each inhibition has been determined from time-course studies.

In this paper, the term "cell wall methylation" is used to describe the transfer of methyl groups from methionine to the hot-water-soluble fraction of the cell wall. The effect of ethionine on the methylation of cold-water-soluble pectin has not been examined in this study.

MATERIALS AND METHODS

Seedlings of *Avena sativa* L. var. Victory for all experiments were grown in the manner of McRae and Bonner (9). The effect of ethionine on cell wall methylation was determined by the following technique, based on Ordin et al (11). Coleoptiles 2.75 to 3.25 cm in length were selected and after the central leaf had been removed, a single 10 mm section was cut from each coleoptile. The apical cut was 3 mm below the tip. The sections were divided into lots of 70 and each lot was then incubated for 3 or 3½ hours in 10 ml of 0.0025 M K-Maleate solution (pH 4.8) which contained C^{14}-methyl labeled L-methionine (2×10^{-5} M). The methionine had a specific activity of 0.145 mC/mg. Each solution also contained one of the following sets of additions: 5 ppm 3-indoleacetic acid (IAA), DL-ethionine (0.05 M), IAA and ethionine, or no additions (control). At the end of the incubation, the sections were rinsed with distilled water, ground rapidly in a mortar and the cell wall debris was separated from soluble material by repeated washing with water on a sintered glass funnel. The pectin fraction of the wall was then isolated by extracting the wall twice for 10 minutes with 2 ml of hot water. An aliquot of the combined extract was then plated out and counted. The amount of saponifiable methyl groups in this extract was determined by ascertaining the loss in radioactivity upon saponification of the extract with hot concentrated NH_3.

The material for the elongation experiments consisted of 5 mm Avena coleoptile sections which still contained the central leaf. These sections were obtained in the manner of McRae and Bonner (9). One lot of 20 sections was immediately plasmolyzed by treatment for 90 minutes in 1 M mannitol and the length of the sections was determined (PLi). The remaining sections were placed in 20 ml of a solution which contained K-Maleate (0.0025 M, pH 4.8), sucrose (1 %) and IAA (5 ppm). Some solutions contained no further additions, others contained either DL-ethionine (0.05 M) or ethionine and ethylenediamine tetraacetic acid (EDTA, 0.001 M). After the desired length of time, sections were removed and measured (Lf). They were then plasmolyzed and remeasured (PLf). Irreversible elongation (PLf-PLi) and reversible elongation (Lf–PLf) were determined.

All manipulations were carried out under dim red light. The temperature during growth of the seedlings and during the course of the experiments was 25 ± 2° C.

RESULTS

The effect of ethionine on cell wall methylation can be seen from the results shown in table I. Experiments 1 and 2 were performed at the laboratory of Professor Bennet-Clark in 1959. Experiment 3 is one of three similar experiments carried out in the laboratory of Professor Bonner in 1956. They were identical to the other two experiments except that the total rather than the saponifiable activity of the hot-water extracts was determined. Ethionine caused a distinct but variable inhibition of cell wall methylation

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TABLE I

EFFECT OF ETHIONINE ON INCORPORATION OF RADIOACTIVITY FROM METHIONINE (METHYL-C^{14}) INTO HOT-WATER-SOLUBLE FRACTION OF CELL WALL

5 *ppm IAA*	0.05 *M ethionine*	EXPT 1 *cpm MeOH/ 70 sections*	EXPT 2 *cpm MeOH/ 70 sections*	EXPT 3 *cpm/10 mg dry cell wall*
+	−	831	740	3,064
−	−	432	630	1,917
+	+	155	60	867
−	+	201	60	874
Incubation time		3½ hr	3½ hr	3 hr
cpm Methionine administered		3.7×10^5	3.7×10^5	3.5×10^5

in all five experiments. Furthermore, in every experiment ethionine completely suppressed the ability of auxin to increase the rate of methyl transfer.

The decrease in methyl transfer induced by ethionine was not due to an inhibition of the uptake of methionine into the tissue. This has been shown in the following way. Sections were incubated for 1 hour in a solution containing methionine-C^{14} but no ethionine. They were then transferred to water, with or without 0.07 M ethionine, and the incorporation of radioactivity into wall pectin was determined after 0, 1, and 2 hours. Cell wall methylation was inhibited by ethionine even under these conditions where ethionine could have had no effect upon the uptake of methionine (fig 1). The inhibition can only be ascribed to an interference with the transmethylation reaction. It should be noted that cell wall methylation was strongly inhibited within 1 hour after application of ethionine.

The inhibition of auxin-induced elongation by ethionine and the reversal of the inhibition by methionine have been confirmed. This is shown in table II. It should be noted that the Avena coleoptile sections used in this experiment were about one-tenth as sensitive to ethionine as were those used by Schrank (12). This may be due in part to the use of DL- rather than L-ethionine.

The effect of ethionine on the time-course of irreversible elongation is shown in figure 2a. In the absence of ethionine, elongation was rapid (due to presence of 5 ppm IAA) and constant. Ethionine had no effect on IE during the 1st hour after its application and produced only a slight inhibition during the subsequent 2 to 4 hours. Thereafter IE was strongly inhibited. EDTA only slightly alleviated this inhibition.

In the absence of ethionine, an increase in IE is accompanied by a proportional increase in ES (5). Upon addition of ethionine, however, there was an immediate decrease in ES (fig 2b). Often the inhibition was even more pronounced than that shown in figure 2b. ES then increased slightly during the next 3 to 4 hours, but thereafter steadily decreased with time. The decrease could be prevented, in part, by addition of EDTA to the medium. If the addition of EDTA occurred at the same time as the addition of ethionine, no inhibition of ES occurred until after 4 to 6 hours. This is even more clearly seen if ES is plotted as a function of IE (fig 2c). This indicates that the immediate decrease in ES was not due to the addition of osmotic solutes to the external medium.

DISCUSSION

Ethionine causes a strong and immediate inhibition of cell wall methylation. Furthermore, ethionine can completely suppress the ability of auxin to cause an increase in the rate of methyl transfer.

The number of radioactive methyl groups in the cell wall at the end of an incubation with methionine (methyl-C^{14}) is equal to the number incorporated by transmethylation minus the number removed from the wall during the incubation period by cell wall demethylation. At the end of the incubation, cell walls of auxin-treated tissues possess a greater number of radioactive methyl groups than do the walls of non-

TABLE II

INHIBITION OF ELONGATION BY DL-ETHIONINE AND ITS REVERSAL BY L-METHIONINE. EXPANSION PERIOD 5½ HRS. ALL SOLUTIONS CONTAINED K-MALEATE (0.0025 M).

TREATMENT	5 PPM IAA	FINAL LENGTH	Δ LENGTH	% INHIBITION
0.05 M mannitol	+	5.47 mm	0.54	...
	−	4.93		
0.03 M ethionine	+	4.99	0.17	69
	−	4.82		
0.03 M ethionine +	+	5.30	0.34	37
0.001 M methionine	−	4.96		

auxin-treated controls. This may be due to either an auxin-induced increase in incorporation or an auxin-induced decrease in decorporation of methyl groups. Glasziou (7) and Adamson and Adamson (1) have suggested that it is an auxin-mediated decrease in decorporation which occurs. If this were so, ethionine, which inhibits only the methylation reaction, would not be affecting the auxin-sensitive reaction. Thus while it would cause a decrease in amount of radioactive methyl groups found in the cell wall, it should not eliminate the effect of auxin. The complete elimination of the auxin-effect indicates that ethionine is blocking the auxin-sensitive reaction which must be the methylation process.

Ethionine also inhibits cellular elongation (12). It has been shown by time studies that this inhibition of total elongation occurs in two distinct phases. The first phase consists of a decrease in ES immediately after adding ethionine. This decrease can be reversed by EDTA. After 3 to 4 hours, the second phase sets in. This consists of strong inhibition of both ES and IE. The inhibition of ES can be partially reversed by EDTA; the inhibition of IE is only slightly reversed. The inhibition of total elongation by ethionine is mainly due to this second phase of inhibition.

How does ethionine cause these inhibitions? The fact that both the inhibition of cell wall methylation and the initial decrease in ES occur immediately after

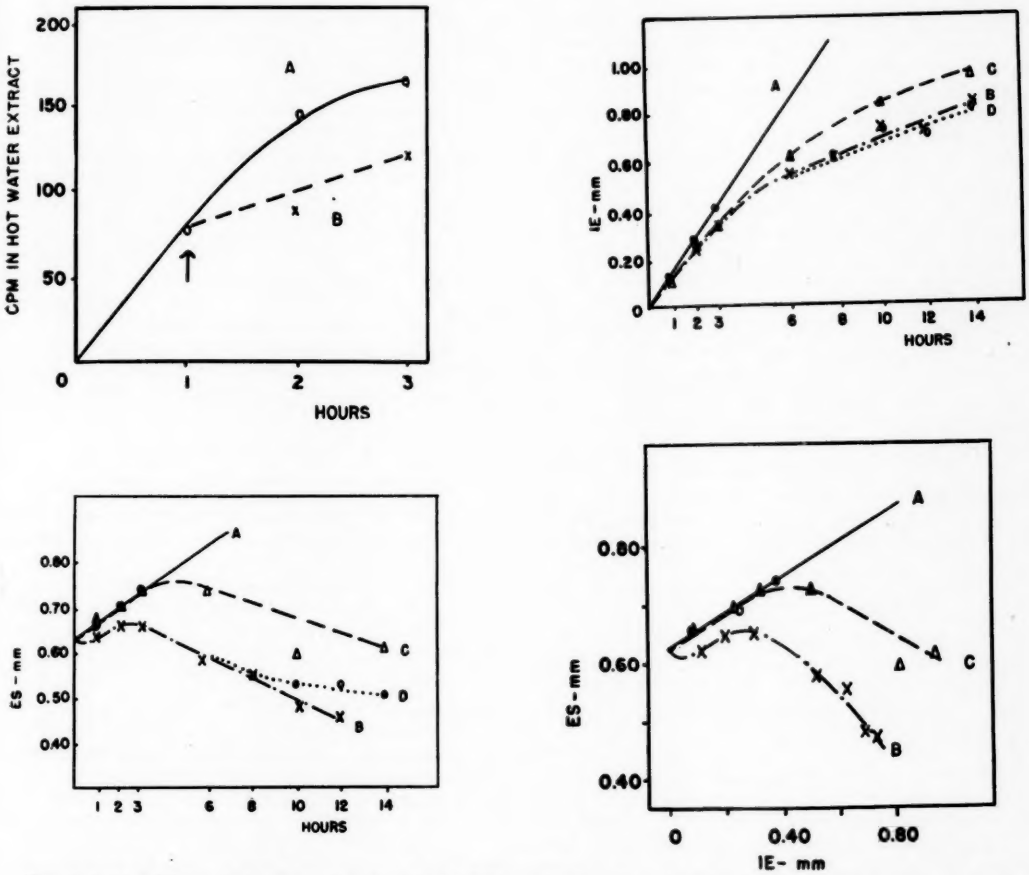

FIG. 1 (*upper left*). A demonstration that the inhibition of cell wall methylation by ethionine is not due to an inhibition of the uptake of methionine into the tissue. Sections incubated with methionine-C^{14} for 1 hour, then placed (at arrow) in water (A) or 0.07 M DL-ethionine solution (B). Incorporation of radioactivity into hot-water-soluble fraction of the wall determined 0, 1, and 2 hours after removal from methionine.

FIG. 2. The effect of ethionine on the time-course of irreversible and reversible elongation. All solutions contained K-maleate (0.0025 M), sucrose (1%) and IAA (5 ppm). Curve A (-○-), no further additions; B (-×-), 0.05 M ethionine; C (-Δ-), 0.05 M ethionine + 0.001 M EDTA; D (.○.), 0.001 M EDTA added after 6 hours. a) (*upper right*) Irreversible elongation, b) (*lower left*) Reversible elongation, c) (*lower right*) Relation between IE and ES

addition of ethionine suggests that the inhibition of transmethylation is the cause of the decrease in ES. This might occur in the following way.

It has been shown that calcium stiffens cell walls (15). Bennet-Clark (2) has suggested that this occurs through formation by calcium of bridges between the carboxyl groups of adjacent pectin chains. The number of these bridges and thus the rigidity of the wall could be limited by the number of free carboxyl groups. Inhibition of cell wall methylation should cause at least a slight increase in the number of free carboxyl groups. This would lead to more calcium bridges and a decrease in elasticity. EDTA, which can chelate free calcium ions, may be reversing this decrease in ES by removing the newly formed calcium bridges.

There are two objections to this theory. Addition of calcium ions, and thus, presumably, the formation of calcium bridges, reduces the plasticity as well as the elasticity of the Avena coleoptile sections (15). If the inhibition of ES by ethionine involves the formation of new calcium bridges, why does not ethionine inhibit IE at the same time? Secondly, Carr and Ng (4) have shown that with wheat coleoptile tissues, EDTA prevents rather than accelerates the loss of calcium from the cell wall. This is not proof, however, that EDTA does not break calcium bridges.

What is the nature of the second phase of the ethionine inhibition? It is not likely that it results from the inhibition of cell wall methylation, for this phase of elongation-inhibition does not manifest itself until 3 to 4 hours after the inhibition of methylation has occurred. Ethionine has been shown to block protein synthesis in plants (16), animals (14), and bacteria (10). The possibility should be considered that ethionine is inhibiting elongation of Avena coleoptiles by interfering with protein synthesis. Evidence to support this idea is obtained from the fact that another amino acid antagonist, canavanine, inhibits auxin-induced elongation in the Avena coleoptile (3). However, auxin-induced elongation can occur in maize (6) and Avena mesocotyl sections (8) in the absence of any increase in protein level. This suggests that only some small portion of protein synthesis must occur in order for elongation to proceed. This would presumably be some enzyme necessary for auxin-action.

Summary

I. The rate of transfer of methyl groups from methionine to the hot-water-soluble fraction of Avena coleoptile cell walls is strongly inhibited by ethionine. The auxin-induced increase in methylation can be entirely prevented by ethionine. This inhibition occurs within 1 hour after adding ethionine.

II. Ethionine causes an immediate inhibition of the reversible elongation of the tissues. Irreversible elongation is strongly inhibited only after 3 to 6 hours. EDTA will reverse the inhibition of reversible but not irreversible elongation.

III. It is suggested that the inhibition of methylation may be the cause of the inhibition of reversible elongation while an inhibition of protein synthesis may lead to the inhibition of irreversible elongation.

Acknowledgment

I wish to express my appreciation to Professor Bennet-Clark for his help and encouragement during the course of this investigation.

Literature Cited

1. Adamson, D. and H. Adamson. 1958. Auxin action on coleoptiles in the presence of nitrogen and at low temperature. Science 128: 532–533.
2. Bennet-Clark, T. A. 1955. A hypothesis on salt accumulation and the mode of action of auxin. In: The Chemistry and Mode of Action of Plant Growth Substances, R. L. Wain and F. Wightman, eds. Pp. 284–294. Buttersworth Scientific Publications, London.
3. Bonner, J. 1949. Limiting factors and growth inhibitors in the growth of the Avena coleoptile. Amer. Jour. Bot. 36: 323–332.
4. Carr, D. J. and E. K. Ng. 1959. The sequestration of calcium from preparations of wheat coleoptile cell walls. Physiol. Plantarum 12: 264–274.
5. Cleland, R. 1959. The effect of osmotic concentration on auxin-action and on irreversible and reversible expansion of the Avena coleoptile. Physiol. Plantarum 12: 809–825.
6. Davison, R. M. 1957. Studies on cell elongation with reference to geotropic effects. Thesis. Univ. of London, King's College, London.
7. Glasziou, K. T. 1957. The effect of 3-indolylacetic acid on the bindings of pectin methylesterase to the cell walls of tobacco pith. Australian Jour. Biol. Sci. 10: 337–341.
8. Hurst, H. M. 1958. Accumulation processes in relation to extension growth. Thesis. Univ. of London, King's College, London.
9. McRae, H. and J. Bonner. 1953. Chemical structure and antiauxin activity. Physiol. Plantarum 6: 485–510.
10. Nomura, M., J. Hosoda, B. Maruo, and S. Akabori. 1956. Studies on amylase formation by *Bacillus subtillis*. Jour. Biochem. (Tokyo) 43: 841–850.
11. Ordin, L., R. Cleland, and J. Bonner. 1955. Influence of auxin on cell-wall metabolism. Proc. Nat. Acad. Sci., U. S. 41: 1023–1029.
12. Schrank, A. R. 1956. Ethionine inhibition of elongation and geotropic curvature of Avena coleoptiles. Arch. Biochem. Biophys. 61: 348–355.
13. Simmonds, S., E. Keller, J. Chandler, and V. du Vigneaud. 1950. The effect of ethionine on transmethylation from methionine to choline and creatine in vivo. Jour. Biol. Chem. 183: 191–195.
14. Simpson, M. V., E. Farber, and H. Tarver. 1950. Studies on Ethionine. I. Inhibition of protein synthesis in intact animals. Jour. Biol. Chem. 182: 82–89.
15. Tagawa, T. and J. Bonner. 1957. Mechanical properties of the Avena coleoptile as related to auxin and to ionic interactions. Plant Physiol. 32: 207–212.
16. Webster, G. C. 1955. Incorporation of radioactive amino acids into the proteins of plant tissue homogenates. Plant Physiol. 30: 351–355.

APPARENT FREE SPACE OF PLANT ROOTS[1]

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Although it is now generally recognized that solutes may permeate some appreciable fraction of the volume of certain plant organs by free diffusion (4, 6, 10, 19), the magnitude and physiological significance of free space remain highly debatable. Free space measurements have been questioned more with regard to the adequacy of removal of superficial solution than for any other reason. The retention of a very thin film, especially on fine roots after blotting or centrifuging may falsely magnify the free space value (20). To obviate this difficulty, the authors have developed a technique for tagging the external medium with a colloid which does not penetrate free space. India ink particles (Higgins American India Ink No. 4415, Waterproof Black, Higgins Ink Company, Brooklyn, N. Y.) are not adsorbed to or repelled from root surfaces under any of the experimental conditions described. This was confirmed by microscopic examination of roots mounted in India ink following a variety of pretreatments including leaching in distilled water and/or equilibration with NaCl solutions. By measuring the amount of India ink carried over on the surface of the root, the volume of root medium superficially retained by the root is determined. The quantity of test solute present in this volume of medium is subtracted from the total quantity of test solute carried over with the root to give the amount of solute in the free space of the root.

Using this technique, the apparent free space values were measured for roots of a number of plant species for which data are available in the literature. In the course of these preliminary studies, variable free space values were found for pea and bean roots depending on experimental conditions. These conditions (duration of washing of roots in distilled water, nature of test solute, and duration of equilibration with solute) were therefore examined critically so that a reasonable explanation of the observed effects could be given.

The term "apparent free space" (AFS) includes a built-in apology: The user concedes that AFS describes a hypothetical partial volume which is a true measure of the free space only if the test solute concentration in free space is the same as that in the external solution with which the plant tissue was equilibrated. In the course of the present investigations, the authors were able to make numerous measurements of the time course of exodiffusion. It was therefore possible to compare the actual rates of exodiffusion with those theoretically expected from cylinders with the dimensions of the plant roots. Reasonable agreement between observed and theoretical values supports the view that: A: Free space does contain the test solute in the same concentration as the solution from which it was absorbed, and B: Free space is probably distributed in the roots according to the basic assumptions made in the calculations.

The rapid equilibration of free space with the external medium suggests that at least many sub-epidermal root cells may participate in ion accumulation on essentially an equal basis with the epidermis. According to the more extreme views of Hylmö (13, 18), free space extends radially through the roots to the water-conducting elements of the xylem thus making it possible for the external medium to move by mass flow through free space into the transpiration stream and up into the top of the plant. Kramer (17) also has suggested that free space may be an interconnecting ramified system extending throughout the plant body. These views make it difficult to account for extreme cases of selectivity in ion absorption, for example, the almost complete exclusion of sodium from the leaves of many plant species (2). Since high transpiration can apparently induce a real increase in ion uptake under some conditions (13), the mechanism for this effect rather than the effect itself is questioned. An alternative to mass flow into the xylem is therefore proposed. If free space extends radially into the root as far as some limiting layer, such as the endodermis (1, 7, 16, 19), a more rapid absorption of water than solute by root cells from free space and particularly by the deeper-lying cells of the free space zone would tend to increase the solute concentration in the free space solution. The external solution would move into free space by mass flow (21, 23), while the outward diffusion of solute would tend to reduce the concentration in free space to that of the external solution. The equilibrium condition may be such (see below) that the endodermal cells and many of the cortical cells absorb solute from a solu-

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tion in free space significantly more concentrated than the external medium, possibly accounting for greater salt uptake. The higher solute concentration in free space under conditions of high transpiration should result in a larger *apparent* free space value.

In estimating the osmotic pressure of root cells by cryoscopic techniques, the free space solution can be an important source of error (3). Whatever solution is present in free space will mix with the vacuolar sap and probably alter its osmotic pressure. It is therefore fittingly retributive that the AFS method be adapted to the measurement of the osmotic pressure of roots. The separation of protoplast from cell wall increases the partial volume of the tissue occupied by the external medium (free space) and this increase in free space can therefore be used to determine the onset of plasmolysis. A sensitive method is described for estimating the osmotic pressure of roots by this technique.

The term "apparent free space" (AFS) will be used to designate that fraction of the root volume into which the external solution appears to diffuse. According to Briggs et al (4, 5, 6), AFS equals "Donnan free space" (DFS) plus "water free space". AFS has been generally used, however, even when the DFS component may be zero (as with mobile anions) and AFS will be so used in the following.

Methods

AFS Method: All plants were grown in water cultures of various types and for varying durations depending upon specific requirements. The excised or intact roots were thoroughly rinsed in tap water, distilled water or in portions of the culture solution and then transferred to a solution of mannitol and/or chloride salts for an equilibrating period which ranged from 15 minutes up to 2 hours in different experiments. The equilibrating solution was changed several times during this period. During the last minute of the equilibrating period, a 1:10 dilution of India ink containing the same concentration of mannitol and/or chloride as the equilibrating solution was added, using 1 ml of the diluted India ink per 20 ml of the equilibrating solution. To facilitate mixing, the roots were momentarily lifted out of the solution while the diluted India ink was mixed in and then dipped several times into the solution which was stirred vigorously each time the roots were lifted out. After mixing and equilibrating for approximately 30 seconds, the roots were allowed to drain for 30 seconds; then were transferred into a beaker of distilled water for exodiffusion. The volume of water was usually some 20 to 50 times the root volume (or fresh wt). During the first minute after transfer, the roots were dipped repeatedly into the water with vigorous intermittent stirring. At the desired time intervals (usually 1, 5, 15, 30, and 60 min), samples of the exodiffusion medium were taken. The optical density (OD) of a 1:50 or 1:100 dilution of the equilibrating solution was determined in the Beckman DU spectrophotometer at 500 mμ[2] as well as the OD of the exodiffusion medium at 1 and 5 minutes. Since the India ink obeys Beer's law in the range of dilutions employed (OD 0.050 to 0.500), the volume of equilibrating solution carried over superficially on the roots may be calculated as follows:

(OD_{eq}) (dilution factor) $\times = OD_{ex}$ $(V_w + \times)$, where eq refers to the diluted equilibrating solution, ex to exodiffusion medium, V_w to ml water used for exodiffusion, and $\times$ equals the milliliters of equilibrating solution carried over into the water on the surface of the roots. Although the first sample of exodiffusion medium was generally withdrawn after 1 minute, in some tests a sample was taken after only 10 or 15 seconds. In all cases, the OD of the initial sample was essentially the same as the OD of later samples indicating a rapid and complete dispersion of the India ink in the water. Macroscopic and microscopic examination of the roots confirmed the quantitative removal of India ink upon dipping the roots into distilled water.

The test-solute (mannitol and/or chloride) concentrations were determined in the equilibrating solution and the exodiffusion medium. The quantity of solute carried over on the root surface was subtracted from the total quantity in the exodiffusion medium to give the free-space solute. This quantity divided by the solute concentration in the equilibrating solution gave the volume of AFS in the root (6) which was then expressed as a percentage of the root volume (i.e., root fresh wt, since the specific gravity of roots was found to be very close to 1.00).

Mannitol and Chloride Methods: Mannitol was determined by a modification of the method described by Butler (7). We found the reaction between periodate and mannitol was completed in 10 minutes at room temperature (25° C). The excess periodate was stable for at least 24 hours and could be estimated at any time by adding KI and titrating the liberated I_2 with standard sodium thiosulfate. The method was otherwise as described by Butler. The modified procedure has the further advantage that once the periodate is added the solution may be stored without change by microbial action. Tests on exodiffusion media from roots not exposed to mannitol solution gave perfect blanks indicating no liberation of periodate-reactive substances from the roots. In most tests the equilibrating solution was 0.035 M mannitol and a 2 or 3 ml aliquot of the 1:100 dilution of the equilibrating solution and similar aliquots of the filtered exodiffusion medium could be analyzed directly.

Chloride was determined by titration with 0.01 *N* $Hg(NO_3)_2$ with 1 % diphenylcarbazone as indicator (26). Using 0.04 *N* chloride equilibrating solutions (usually mixtures of NaCl and $CaCl_2$), 10 ml aliquots

[2] Since wave length is not critical, a colorimeter of equivalent precision may be used in place of the Beckman spectrophotometer.

of the exodiffusion media or of the diluted equilibrating solutions could be titrated with an accuracy of 1 or 2% using a Koch 2 milliliter buret. The India ink did not alter the blank or interfere with either the mannitol or chloride determinations.

LIMITATIONS OF AFS METHOD AND SOURCES OF ERROR: Dilutions of India ink in distilled water are stable if the concentration of added polyvalent cations does not exceed 3 meq/l. Four meq/l Ca causes the ink to flocculate in about half an hour. When chloride was used in equilibrating solutions, the maximum level of calcium employed was therefore 0.003 *N*, the balance of the cations being Na.

Most roots lose enough cells, especially root cap cells, during rinsing to contribute slightly to the OD reading of the exodiffusion medium. Pre-rinsing of the roots is therefore necessary to hold sloughing of cells during exodiffusion to a minimum.

RESULTS AND DISCUSSION

AFS VALUES; LEACHING EFFECTS: In order to compare free space values determined by the India ink tagging method with data in the literature, the free space in the roots of a number of plant species grown on dilute nutrient solution (BN/2, table II) was determined. Except for the beans, 10- to 20-day old plants from a controlled environment room were used. The bean roots were obtained by excising the distal 10 to 15 cm of roots from full-grown greenhouse cultures.

All roots were washed in several changes of distilled water for a period of several hours and then equilibrated for varying periods with 35 mM mannitol and/or 40 mM NaCl. The results are summarized in table I.

The free space values for Gramineae (table I, A through F) fall mostly in the range 20 to 30 percent

TABLE I

AFS IN ROOTS OF CONTROL-ROOM GROWN SEEDLINGS AND IN BEAN ROOTS FROM GREENHOUSE CULTURES

	MATERIAL*			MANNITOL ONLY, 0.035 M	MANNITOL + 0.040 *N* NaCl	
			T_{EQ}** *Min*	AFS_M %	AFS_M %	AFS_{Cl-} %
A.	Barley, Atlas 10-day old seedlings	Intact Excised	15 15	23.0, 24.9 21.1, 31.0		
B.	Barley, Atlas 8-day old seedlings	Intact Excised Intact	15 15 150		30.1 46.6 25.4	24.2 38.7 32.9
C.	Barley, Atlas 14-day old seedlings	Excised	15 30	32.6 24.8		
D.	Wheat, Ramona 14-day old seedlings	Excised	120	31.7	30.6	25.0
E.	Tall Wheatgrass, 15-day old seedlings	Excised	120	25.2		
F.	Corn, Hickory-King 19-day old seedlings	Excised	120	20.5	20.4	18.7
G.	Pea, Alaska 15-day old seedlings	Excised	120	7.4	14.9	11.6
H.	Bean, Red Kidney Mature plant	Excised	60 60	6.5 9.9, 7.0		
I.	" "	Excised	20 120		No mannitol	9.7 16.5
J.	" "	Excised	15 150		8.5 16.0	6.1 13.5

* A, B, C: *Hordeum vulgare* L.; D: *Triticum vulgare* Vill.; E: *Agropyron elongatum* Host.; F: *Zea mays* L.; G: *Pisum sativum* L.; H, I, J: *Phascolus vulgaris* L.

** Equilibration time.

and are therefore in good agreement with values in the literature for barley and wheat (7, 9, 16, 18). In the well-aerated, relatively shallow water cultures, moderate to strong root-hair development was observed in all Gramineae except corn (cf. Butler, 7). The epidermal cells of barley roots without root hairs were readily plasmolyzed by 0.3 or 0.5 M mannitol, and therefore the entire volume of such cells could not be free space. Cells with root hairs, however, could not be plasmolyzed, indicating that they were readily permeated by the mannitol. Since cells with and without root hairs alternated in the case of barley, striking comparisons could be made. It appears, therefore, that part of the measured free space of Gramineae may reside in the highly permeable epidermal cells with root hairs. Whether this permeability was inherent or the result of injury to the delicate root hairs cannot be stated. Corn roots, however, which at the most bore only incipient root hairs, still had approximately 20 % apparent free space.

Unlike the findings of Jacobson et al (16), the AFS of excised roots was not consistently greater than the AFS of intact roots (table I, A and B). Comparable values for intact and excised roots were generally obtained, although it should be emphasized that the excised roots were rather long so that the ratio of cut surface to root length was small.

In contrast to the Gramineae, the pea and bean roots had markedly less free space. Root hairs in these species were at the most rudimentary and the epidermal cells could be readily plasmolyzed. While similar values for free space of gramineous roots were obtained by either chloride (NaCl) or mannitol, large differences were noted under some conditions for pea and bean roots. With mannitol alone, moderate values of 6 to 10 % were obtained (table I, G and H; cf. 12, 13). After equilibration with NaCl solution for 2 hours, the AFS values were as high as 16 %. A 15- or 20-minute equilibration period with NaCl solutions did not result in high free space values (table I, I and J). If the conditions for high free space were satisfied (i.e., equilibration with NaCl for 2 hrs), then the AFS measured by mannitol, simultaneously present in the equilibrating solution, was also increased (J, 150 min). These curious findings for roots washed several hours in distilled water before equilibration were confirmed and elaborated by additional experiments.

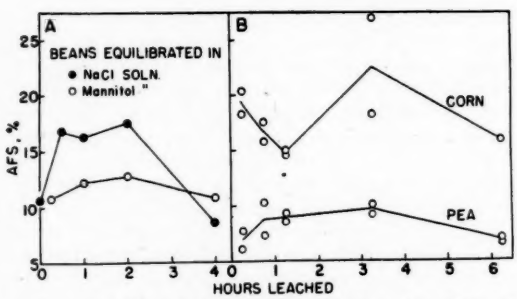

FIG. 1A. Mannitol and chloride values for AFS of leached, excised bean roots. NaCl series: Roots leached in several changes of distilled water for total leaching time indicated, then equilibrated 15 minutes in 35 mM NaCl. Mannitol series: Roots leached and equilibrated in 3 changes of 35 mM mannitol for total leaching times indicated.

FIG. 1B. Effect of leaching on AFS of intact pea and corn roots. Roots leached in distilled water for 0 to 6 hours and then equilibrated 15 minutes in 35 mM mannitol. Leaching time includes the 15-minute equilibration with mannitol.

Because the NaCl-effect on the AFS of bean and pea roots suggested that leaching of salts by preliminary rinsing of roots was probably a factor, the effect of leaching on AFS was examined. Bean and pea roots leached in successive changes of either distilled water or a mannitol solution showed only a moderate increase of about 2 to 3 %-units in AFS as determined by mannitol (fig 1, A and B). However, when NaCl was used to measure AFS of leached bean roots, larger increases of 6 to 7 %-units like those previously observed did occur (fig I A). Furthermore, this effect was apparent in roots leached 0.5 to 2 hours, even though equilibrated with 35 mM NaCl for only 15 minutes. The longer exposures to NaCl of 2 hours required in the earlier tests (table I, I and J) are now seen to be characteristic of roots leached and washed for more than 2 hours, the practice in the earlier experiments. The 15- or 20-minute equilibration of such roots with NaCl yields a low AFS of 5 to 10 % and longer equilibration of about two hours is necessary for the high AFS value. Whether measured by mannitol or chloride (short equilibration), AFS goes through a maximum associated with moderate leaching and then returns to the initial low values after about four hours of leaching.

Corn roots differ markedly from pea and bean roots in their response to leaching. The procedure with corn was entirely comparable to that for pea, but with corn, short periods of leaching (up to 1 hr) tend to decrease rather than increase AFS. A rather abrupt and variable increase in AFS was noted after 3 hours of leaching; but after 6 hours of leaching, AFS decreased again to the lower level.

Analysis of leachates from bean and milo (*Sorghum vulgare* Pers.) roots revealed a loss of Ca, K, and some Mg from the roots. Versenate titration was employed to determine Ca and Mg and flame photometry for K (26). Exchangeable cations in corn, pea, and wheat roots were also determined by extraction with 0.02 *N* acetic acid for several successive 5-minute periods. The readily diffusable free-space cations were first removed by two or three 30-second rinses in water. The exchangeable Ca amounted to 7 to 15 meq/100 g dry weight of roots or about 0.35 to 0.75 meq per 100 g fresh weight (cf. 27). About half of this Ca (3.7 meq out of a total of 6.5) was lost by corn roots during 30 minutes of leaching in distilled water and in other leaching studies

TABLE II

EFFECT OF CULTURE SOLUTION CONCENTRATION ON AFS OF INTACT PLANT ROOTS, EQUILIBRATED IN 0.040 *N* CHLORIDE FOR INDICATED TIMES, T_{EQ}

MATERIAL	T_{EQ}	RELATIVE CULTURE SOLUTION CONC			
		2 BN	BN	½ BN	⅛ BN
	Min		*AFS %*		
Bean, Red Kidney	30	9.6	5.6	7.5	...
	60	10.3	7.2	10.6	...
Corn, Golden Cross Bantam	30	18.2	15.8	16.4	17.1
	60	16.3	14.8	15.7	17.2
Pea, Alaska	15	7.3	4.5	6.6	7.2

Base nutrient solution concentrations in millimoles/l: $Ca(NO_3)_2$, 5.0; KNO_3, 6.0; $MgSO_4$, 3.0 and KH_2PO_4, 1.0; also 1 ppm Fe as ferric citrate and 0.5 ppm B and Mn as H_3BO_3 and $MnSO_4$, respect. Demineralized water used and pH adjusted to 5.5.

large fractions of the exchangeable Ca were lost during the course of several hours of leaching. No well-defined exchangeable K fraction was found, and leaching losses of K, while large, did not appear to be correlated with changes in free space. A reasonably well-defined exchangeable Mg fraction of 5 to 10 meq/100 g dry weight of roots was demonstrated by acetic acid extraction but only a small fraction of this Mg appeared in water leachates.

The foregoing observations on the loss of cations and changes in AFS with leaching may be related as follows: The increase in AFS with short periods of leaching is associated with the loss of exchangeable cations (primarily Ca) from the roots. Recent findings indicate that both AFS and exchange sites for cations are largely if not entirely in the cell walls (8, 11, 22). While Ca is being lost, the cell wall appears to be in a highly metastable state so that great changes in wall structure as indicated by AFS values may occur. If salt is absent from the equilibrating solution, only small increases in AFS are noted, but if salt is present, much larger increases occur. The loss of exchangeable cations may be expected to increase colloidal hydration and therefore the AFS. The presence of NaCl should promote the loss of exchangeable calcium. The calcium acting as cross-links between carboxyl groups of adjacent fibrils may be particularly important in controlling wall structure and volume (7, 24, 25). The accelerated release of calcium may therefore result in the magnified increase of AFS in the presence of NaCl. The decrease in AFS after 4 or more hours of leaching suggests that structural modifications of the cell wall have taken place, perhaps the methylation of the carboxyl groups. The cell wall is thus stabilized at a lower volume and is not susceptible to large expansion by brief treatment with NaCl. However, the 2-hour treatment with NaCl can apparently cause reversion to the high AFS state. The salt may perhaps favor demethylation.

The failure to observe consistent salt effects on AFS of gramineous roots may be due to differences in root structure or possibly to a greater activity of the mechanism for restabilization of wall structure. This may result in an appreciable shortening of the period of metastability. The abrupt and relatively short-lived rise in AFS for corn roots leached for 3 hours supports this view.

Since partial depletion of diffusible salt (exchangeable cations) appears to affect the AFS of some roots so strongly, a number of plant species were grown in serial dilutions of nutrient solution ranging from a total concentration of 46 meq/l (double strength) to 2.9 meq/l (table II). AFS was measured following equilibration with 0.038 *N* NaCl + 0.002 *N* $CaCl_2$; the roots were first rinsed thoroughly in their own culture media. Pea and bean grew poorly in the most dilute solution and only those treatments with healthy roots were tested. Minimal AFS values were obtained consistently for roots grown in single-strength BN solution and somewhat higher values for roots from the more dilute and more concentrated solutions (table II). These effects do not resemble the response of partially leached roots to salt. The wall structure of the roots apparently adjusts to a wide range of salt concentrations during the course of growth. Determination of exchangeable cations by leaching roots with 0.02 *N* acetic acid showed a somewhat lower content of exchangeable Ca in pea and wheat roots from dilute solutions than from the more concentrated solutions. The presence of 2 meq/l calcium in the equilibrating solution in addition to the 38 meq/l sodium should not have obscured any salt response inasmuch as bean roots leached 2 hours showed high AFS values of 15.2, 16.3, and 25.3 % when subsequently equilibrated 2.5 hours with 0.035 *N* chloride containing 0, 1, and 3 meq/l Ca, respectively. Indeed, the 3 meq/l Ca solution was most effective in producing the salt response. This also suggests that specific Ca bonds, such as cross-linkages, which could

be broken by other Ca ions as well as by Na, were primarily controlling cell wall structure and therefore free space.

HALF-TIMES FOR EXODIFFUSION: The reality of free space and its radial extent in roots are attested to by agreement between actual and theoretical exodiffusion rates. The data for individual experiments will not be presented, but a summary of the results is given in table III. Despite the fact that the diffusion coefficient for chloride salts is generally more than twice that of mannitol [1.4×10^{-5} for NaCl vs 0.6×10^{-5} for mannitol (15)], exodiffusion rates for chloride and mannitol are similar. The diffusion coefficient for chloride through the charged microfibrillar network is apparently reduced to that of mannitol. One may compare the observed time course of exodiffusion with theoretical diffusion from a cylinder of infinite length where the average concentration, C_{av}, remaining at time, t, in relation to C_s and C_0, the external and initial internal concentrations, respectively, is given by the series (14, p 278):

$$\frac{C_{av} - C_s}{C_0 - C_s} = 4\left(\frac{1}{z_1^2} e^{-Dtz_1^2/R^2} + \frac{1}{z_2^2} e^{-Dtz_2^2/R^2} + \ldots\ldots\right), \quad \text{II}$$

z_1 and z_2 are series coefficients. C_s, the external concentration, is only 1 to 2 % of C_0 in these experiments and is moreover almost constant with time, most of the solute being carried over on the surface of the root. The left-hand member of the equation therefore may be taken as $\frac{C_{av}}{C_0}$, the fraction of the ultimately diffusable solute still remaining in the root at time, t. Although this equation describes diffusion from a solid, not a hollow, cylinder, the discrepancy is very minor because of the small root diameters and the tortuosity of the diffusion path.

Assuming a diffusion coefficient, D, of 10^{-5} cm^2/sec, values for R, the radius of the cylinder, were calculated for each time interval at which C_{av} was determined. R increases progressively in each case with increasing t, due to the range of root radii represented in any root system. For beans and corn, the R values in cm based on the mannitol data were:

	Min			
	1/4	*1*	*5*	*30*
Beans	0.027	0.044	0.084	0.17
Corn, Hickory-King	...	0.079	0.130	0.21
Corn, Golden Cross Bantam	...	0.068	0.117	0.19

The finest lateral rootlets of red kidney bean measure just about 0.027 cm in diameter, while the larger roots on which these laterals are borne are about 0.061 cm. The tortuosity of the diffusion path for root cells isodiametric in cross section makes the diffusional distance about twice the radial distance[3]. Using the bean-root mannitol data in table III, the contribution of roots of different diameters to the total free space of the root system was computed. It was obvious to begin with that the diffusional radius of the smallest roots had to be less than 0.027 cm because such roots would reach 78 percent of equilibration in 15 sec, the value actually found for the whole root system. The finest roots of course have to achieve better than average equilibration to balance the slower equilibration of larger roots. The innermost limit of free space was set at 2/3 of the way into the root, the approximate location of the endoder-

[3] Actually, the ratio of distances for cylindrical cells of the cortex would be $\pi/2$.

TABLE III

TIME COURSE OF EXODIFFUSION. PERCENT OF TOTAL EXODIFFUSION AT INDICATED TIME INTERVALS

MATERIAL	SOLUTE*	No. OBS.	TIME, MIN				
			¼	1	5	30	60
Bean, Red Kidney	M	11	78.0**	88.6	93.9	98.2	99.6
Bean, Red Kidney	Cl	18	75.6**	85.8	90.6	97.9	100
Corn, Hickory King	M	8	...	59.9	75.1	93.4	98.8
Corn, Hickory King	Cl	7	...	64.9	77.2	93.1	98.8
Corn, Golden Cross Bantam	M	12	...	57.5	80.4	96.6	100
Corn, Golden Cross Bantam	Cl	12	...	60.5	76.5	94.1	100
Corn, Golden Cross Bantam	Cl	8	...	62.3	76.0	93.9	100
Barley, Atlas	M	3	70.0	80.0	89.0	100	...
Wheat, Ramona	M & Cl	3	...	83.8	90.7	96.0	100
Pea, Alaska	M	2	...	72.2	80.4	95.6	98
Cotton, Acala 4-42***	M	4	...	85.6	93.8	100	...

* M = mannitol, Cl = chloride
** Avg 3 determinations only
*** *Gossypium hirsutum* L.

mis, so that for the roots of 0.027 cm diameter the diffusional radius, R, becomes $0.0135 \times 2 \times 2/3$ or 0.018 cm and for the larger roots of 0.051 cm diameter, R equals 0.041 cm. Since the still larger roots near the stem are variable in size, several values for the R of these roots were tried. The degree of diffusional equilibration was calculated for each root size at 15 seconds, 1 minute, and 5 minutes using equation II and the three equations for the three times become:

$$\begin{aligned} 0.952\ X + 0.587\ Y + 0.323\ Z &= 0.780 \\ X + 0.912\ Y + 0.366\ Z &= 0.886 \\ X + \quad Y + 0.552\ Z &= 0.939 \end{aligned} \qquad \text{III}$$

X, Y, and Z denote the fraction of the total free space represented by roots having diffusional radii of 0.018, 0.041, and 0.200 cm, respectively. The coefficients for these terms represent the theoretical degrees of equilibration reached by such roots at the given times and the right hand members are the average degrees of equilibration observed for the whole root system at these times (table III). Solution of these simultaneous equations indicated 63 percent of the free space associated with the finest roots, 21 percent with the larger roots on which the fine roots are borne and 18 percent with the coarser roots having larger diameters still (assumed to be 0.300 cm in this case). Although not required to add up to 100 % by the equations, the values do total 102 % indicating that the results are reasonable. The solution of the equation for the 30-minute interval indicates 101 percent equilibration in fair agreement with the observed 98 %. An alternative solution assuming a diffusional radius of 0.100 cm for the largest root fraction yields values of 67, 18, and 10 % in order of increasing root size for the three root fractions. The observed diffusional rates are therefore readily explainable on the basis of the actual root dimensions. If constant geometry is assumed for roots of different sizes (the free space extending into any root the same fraction of the root radius, actually the case for the finest and medium-sized bean roots), then the distribution of free space among roots of different sizes also becomes the size distribution of the root system; i.e., 63 % of the total root weight consists of roots having diameters of 0.027 cm, etc. While not actually checked by dissection, these relationships seem reasonable.

The possibility that a small fraction of the total diffusible solute may be diffusing out of the cytoplasm or even the vacuole, for which processes the half times would be reckoned in hours or even days (8, 22) cannot be completely discounted. This effect would also cause an apparent increase in the calculated diffusional radius with time.

The half-times for exodiffusion were determined by extrapolation using the data in table II and are approximately 2 seconds for bean, barley, wheat, and cotton, 12 seconds for pea, and 25 seconds for corn. Very few estimates of diffusional half-times have been made for plant roots (18).

TRANSPIRATION RATE AND AFS: If transpiration or any other water use causes an uptake of water from free space by the cortical and endodermal root cells and a mass flow of solution into the cortex (21, 23), an accumulation of solute in free space must result. The degree of solute accumulation can be readily calculated from the diffusion equation (14, p 37) for conditions of dynamic equilibrium. In this calculation, the endodermis is taken as the innermost limit of free space on the assumption that the Casparian strip effectively prevents further inward movement into the root through cell walls (free space). An uptake of solution equal to fresh weight of the root in an hour (table IV) is equivalent to 0.002 ml per hour per cm of root 0.5 mm in diameter. The exodiffusion per second is therefore:

$$\frac{0.002}{3600} = \frac{2\pi D\ (C_1 - C_0)}{\ln r_0 - \ln r_1} \qquad \text{IV}$$

where D is the diffusion coefficient, C_1 and C_0, the concentrations at radial distances r_1 and r_0, respectively, where r_0 = root radius and $r_1 < r_0$. Solving for the concentration at a point, r_1, 2/3 of the way into the root (the approximate location of the endodermis) and using 10^{-5} for the diffusion coefficient, $C_1 - C_0 = \frac{1.1}{113}$. But since free space constitutes only in the order of 10 to 30 percent of the total root volume, the cross sectional area for diffusion is correspondingly less than the cylinder area. If the free space is taken to be 20 percent, then about half or 10 percent will be radially or transversely-oriented space and half will be tangentially-oriented (for elongated cells). With the same amount of diffusion occurring across 10 percent of the cross-sectional area, $C_1 - C_0$ must be increased ten fold and C_1 becomes 10 percent greater than C_0. If tortuosity of the diffusion path is now taken into account by assuming cells to be isodiametric in transverse section, then this doubling of the diffusion path (See footnote 3), makes C_1 14 percent greater than C_0. The *average concentration* in free space should therefore be approximately seven percent greater than that of the external medium under these conditions.

Higher transpiration rates, lower free space percentages, and larger root radii would increase the concentration factor in free space. Transpiration rates up to 3.5 times as great and free space values half as large as those employed in the calculation were in fact observed (table IV). A combination of the extremes should therefore theoretically increase the concentration in free space up to 7 times the 14 percent estimated above or nearly double the external concentration. The *average concentration* in free space would in this case be 40 % greater than that of the external medium.

Appreciable increases in solute concentration in free space are therefore possible, under these conditions.

Five different experiments, summarized in table IV, were performed to determine the effect of transpiration rate on AFS. Four-plant cultures were

TABLE IV

AFS IN ROOTS OF RAPIDLY AND SLOWLY TRANSPIRING PLANTS. AFS VALUES CALCULATED FOR CHLORIDE AND MANNITOL PRESENT SIMULTANEOUSLY IN EQUILIBRATING SOLUTIONS, EXCEPT FOR MILO TESTED WITH CHLORIDE ONLY. CHLORIDES IN EACH CASE CONSISTED OF 2 MEQ/L $CaCl_2$, BALANCE BEING NaCl

MATERIAL	REPS.	SOLUTION	HIGH TRANSPIRATION AFS±SE	HIGH TRANSPIRATION T.R.*	LOW TRANSPIRATION AFS±SE	LOW TRANSPIRATION T.R.*
Corn	3	(0.020 M Mannitol	25.2 ± 5.00	...	22.4 ± 1.86	...
Hickory King		(0.020 *N* Cl^-	26.1 ± 6.70		24.5 ± 2.46	
Cotton	2	(0.020 M Mannitol	16.2 ± 2.05	3.51	14.6 ± 1.06	0.34
Acala 4-42		(0.020 *N* Cl^-	19.6 ± 0.05		16.3 ± 3.40	
Bean	3	(0.010 M Mannitol	9.0 ± 0.56	2.28	9.6 ± 0.80	0.29
Red-Kidney		(0.016 *N* Cl^-	8.2 ± 0.75		9.9 ± 1.37	
Corn	3	(0.010 M Mannitol	11.8 ± 1.90	0.90	8.7 ± 0.31	0.23
Golden Cross Bantam		(0.016 *N* Cl^-	13.5 ± 0.86		10.4 ± 0.97***	
Milo** Double Dwarf	3	0.016 *N* Cl^-	10.6 ± 0.97	1.35	11.3 ± 0.60	0.18

* Transpiration rate, g water per g fresh root per hour.
** *Sorghum vulgare* Pers.
*** Significant at odds of 19 : 1.

grown in full-strength BN solution (table II) in the greenhouse to a suitable size. Some of these cultures were allowed to equilibrate for 1 or 2 hours in the greenhouse in test solutions under conditions favoring maximal transpiration, while others equilibrated in the laboratory where transpiration was much less (table IV). The AFS was then measured in the usual manner, the greenhouse treatments being transferred into the laboratory once exodiffusion had been initiated. The results (table IV) indicate no statistically significant effect of transpiration on AFS except in the case of the Golden Cross bantam corn where odds for the chloride values were 23 to 1 in favor of greater AFS for the rapidly transpiring plants. In other cases (Hickory King corn and cotton), the mean AFS values were also greater for the rapidly transpiring plants but results were not statistically significant. Excessive variability in AFS between some of the replicates greatly decreased the chances for observing significant effects. In addition, manipulation of the plants according to the standard procedures for free space measurements may have checked transpiration and permitted loss of accumulated solute.

Recently, Kylin and Hylmö (18) have reported a consistently higher AFS for rapidly transpiring wheat plants on dilute salt solutions than for slowly transpiring plants. They attached no significance to this observation. With higher concentrations of salt, the AFS still tended to be somewhat greater for the rapidly transpiring treatments.

DETERMINATION OF OSMOTIC PRESSURE AT INCIPIENT PLASMOLYSIS BY AFS PROCEDURE: The root systems of two bean cultures were each divided into eight or more lots of approximately ten grams that were as nearly representative as possible. The roots were thoroughly rinsed in the medium in which they had grown, and one series was then equilibrated for 15 minutes in graded concentrations of NaCl, the second series in mannitol (fig 2). The usual pro-

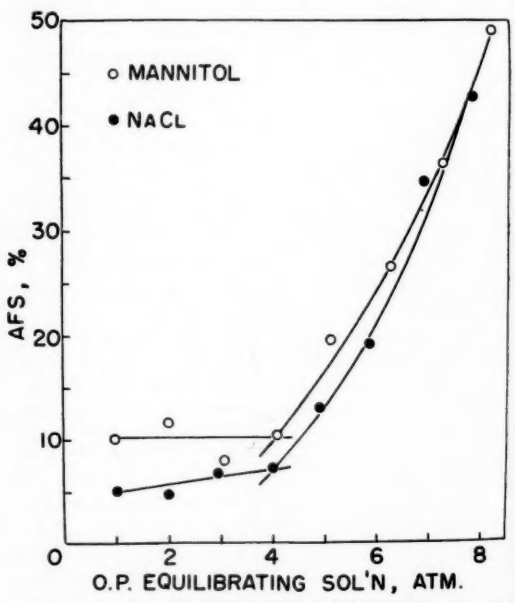

FIG. 2. The effect on AFS of equilibrating excised bean roots in NaCl and mannitol solutions of graded osmotic pressures. Incipient plasmolysis at 4.1 atm causes an abrupt increase in AFS.

cedure was then followed for estimating AFS by means of chloride and mannitol, respectively, using 15-minute exodiffusion periods. The plots of AFS against OP show that an abrupt increase in AFS occurred at 4.1 atm thus fixing with considerable precision the osmotic pressure at which incipient plasmolysis occurred. While the method using NaCl is quicker because of the greater ease of measuring chloride, the mannitol modification is less subject to error resulting from solute uptake or loss by the tissue. Because of the high concentrations of salt involved (24 meq/l per atm), uptake will generally be a negligible factor, but in plants cultured on chloride-containing media, loss of accumulated chloride may affect the results.

The precision of this method appears to be quite good. It is usually difficult to estimate osmotic pressure any closer than 1 atm by observing incipient plasmolysis in roots. Direct observation is usually limited to epidermal and closely underlying cortical cells of the finer lateral rootlets only. The free space method yields an estimate for all roots restricted only by the radial extent of free space in the roots. Finally the results appear to be in good agreement with measurements by the usual cryoscopic method for roots grown in dilute nutrient solutions. Two portions of the root system that was used in the mannitol experiment were blotted dry immediately upon removal from the culture solution. Two other portions were rinsed 2 minutes in several changes of distilled water, then blotted. After freezing and thawing, the sap was expressed and OP determined cryoscopically. The unrinsed roots gave 4.87 and 4.12 atm, the rinsed roots 4.14 and 4.24 atm. The deviations between cryoscopic and plasmolytic values in the case of roots grown on more concentrated media will be reported elsewhere.

Although estimates of OP at incipient plasmolysis are the same for the NaCl and mannitol series, the obvious discrepancies in AFS values between the two series require some comment. The lower AFS values with chloride at the lower concentrations compared to mannitol and the convergence of the curves at higher concentrations strongly suggest the presence of a Donnan free space containing fixed anions from which mobile anions but not the neutral mannitol molecules tend to be excluded (4, 5, 6). As the concentration of mobile anion is increased, more can penetrate the DFS, causing the convergence of the curves. However, such results were *not* consistently obtained in the many other experiments in which mannitol and chloride were simultaneously present in the equilibrating solution affording a much more precise measure of DFS than the data in figure 2. In table I, most of the AFS values by Cl appear to be lower than the simultaneously measured mannitol values, but the reverse appears to be true in table IV. In those tests in which exodiffusion was continued for 2 hours (table I), a slight reabsorption of chloride by the roots was evident during the 2nd hour, causing the lower AFS values. Considering all the pertinent data, one must conclude that no DFS has been demonstrated and that the data in figure 2, determined on separate roots, may reflect initially different free space values rather than a DFS compartment. Since the DFS has been estimated to be only some 10 % of the AFS in beet tissues (5), the possibility of a small and difficultly measurable DFS compartment in plant roots cannot be excluded.

Summary

India ink, a colloid, was used to tag the external medium and distinguish between superficially retained solution and free space solution in plant roots. Using this technique with mannitol and chloride as the test solutes, AFS values in reasonable agreement with those in the literature for a variety of plant species were obtained. The AFS of pea and bean roots was found to change during leaching of the roots and especially so, if salt solutions were subsequently used in estimating AFS. This effect of leaching seems attributable to a loss of exchangeable calcium from the roots which by cross-linking carboxyl groups maintained a certain structure in the cell walls.

Observed rates of exodiffusion of solutes from free space agreed well with theoretical diffusion from cylinders having the dimensions of bean roots. Tortuosity of the diffusion path and a radial extent of free space 2/3 of the way into the root were allowed for in the calculation.

An accumulation of solutes in free space is postulated as water is selectively absorbed by cortical and endodermal cells under the influence of transpiration. Calculations indicate that as much as a doubling in solute concentration could have theoretically occurred under observed conditions. This may account for increased salt uptake by plants under conditions of high transpiration. Experiments on the effects of transpiration on AFS values were inconclusive, although a tendency toward increased AFS under conditions of high transpiration was noted.

The osmotic pressure of roots at incipient plasmolysis can be readily determined by measuring the AFS with mannitol or NaCl solutions of graded concentrations. The OP of bean roots was found to be 4.1 atm, in good agreement with cryoscopic determinations of expressed root sap.

Acknowledgment

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Literature Cited

1. Arnold, A. 1952. Über den Funktionsmechanismus der Endodermiszellen der Wurzeln. Protoplasma 41: 189–211.
2. Bernstein, L., J. W. Brown, and H. E. Hayward. 1956. The influence of rootstock on growth and

salt accumulation in stone-fruit trees and almonds. Proc. Amer. Soc. Hort. Sci. 68: 86–95.

3. Bernstein, L. and H. E. Hayward. 1958. Physiology of salt tolerance. Ann. Rev. Plant Physiol. 9: 25–46.
4. Briggs, G. E. 1957. Some aspects of free space in plant tissue. New Phytol. 56: 305–324.
5. Briggs, G. E., A. B. Hope, and M. G. Pitman. 1958. Exchangeable ions in beet disks at low temperatures. Jour. Exp. Bot. 9: 128–141.
6. Briggs, G. E. and R. N. Robertson. 1958. Apparent free space. Ann. Rev. Plant Physiol. 8: 11–30.
7. Butler, G. W. 1953. Ion uptake by young wheat plants. II. The "apparent free space" of wheat roots. Physiol. Plantarum 6: 617–635.
8. Diamond, J. M. and A. K. Solomon. 1959. Intracellular potassium compartments in *Nitella axillaris*. Jour. Gen. Physiol. 42: 1105–1121.
9. Epstein, E. 1955. Passive permeation and active transport of ions in plant roots. Plant Physiol. 30: 529–535.
10. Epstein, E. 1956. Mineral nutrition of plants: Mechanisms of uptake and transport. Ann. Rev. Plant Physiol. 7: 1–24.
11. Dainty, J. and A. B. Hope. 1959. Ionic relations of cells of *Chara australis*. I. Ion exchange in the cell walls. Australian Jour. Biol. Sci. 12: 395–411.
12. Hope, A. B. and P. G. Stevens. 1952. Electric potential differences in bean roots and their relation to salt uptake. Australian Jour. Sci. Res. Ser. B. 5: 335–343.
13. Hylmö, B. 1953. Transpiration and ion absorption. Physiol. Plantarum 6: 333–405.
14. Ingersoll, L. R., O. J Zobel, and A. C. Ingersoll. 1954. Heat Conduction. 325 pp. University of Wisconsin Press.
15. International Critical Tables. 1929. Vol. 5. 1st Ed. McGraw-Hill, New York.
16. Jacobson, L., R. J. Hannapel, and D. P. Moore. 1958. Non-metabolic uptake of ions by barley roots. Plant Physiol. 33: 278–282.
17. Kramer, P. J. 1957. Outer space in plants. Science 125: 633–635.
18. Kylin, A. and B. Hylmö. 1957. Uptake and transport of sulphate in wheat. Active and passive components. Physiol. Plantarum 10: 467–484.
19. Laties, G. G. 1959. Active transport of salt into plant tissues. Ann. Rev. Plant Physiol. 10: 87–112.
20. Levitt, J. 1957. The significance of "apparent free space" (A.F.S.) in ion absorption. Physiol. Plantarum 10: 882–888.
21. Lopushinsky, W. 1959. Relationship of ion uptake to mass flow of water through roots. Plant Physiol. 34 suppl.: xx.
22. Mac Robbie, E. A. C., and J. Dainty. 1958. Ion transport in *Nitellopsis obtusa*. Jour. Gen. Physiol. 42: 335–353.
23. Mees, G. C. and P. E. Weatherley. 1957. The mechanism of water absorption by roots. II. The role of hydrostatic pressure gradient across the cortex. Proc. Royal Soc., B. 147: 381–391.
24. Ordin, L., R. Cleland, and J. Bonner. 1957. Methyl esterification of cell wall constituents under the influence of auxin. Plant Physiol. 32: 216–220.
25. Tagawa, T. and J. Bonner. 1957. Mechanical properties of the Avena coleoptile as related to auxin and to ionic interactions. Plant Physiol. 32: 207–212.
26. U. S. Salinity Laboratory Staff. 1954. Diagnosis and improvement of saline and alkali soils. U.S. Dept. Agr. Handbook 60, 160 pp.
27. Williams, D. E. and N. T. Coleman. 1950. Cation exchange properties of plant root surfaces. Plant and Soil 2: 243–256.

INTERRELATIONSHIPS OF CATALASE, PEROXIDASE, HEMATIN, AND CHLOROPHYLL [1]

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The formation of chlorophyll in the plant has long been known to depend upon an adequate supply of iron and, although it contains magnesium in its porphyrin group, the striking similarity between the prosthetic groups of chlorophyll and hemoglobin allows similar biosynthetic pathways (14) to be postulated.

The iron-porphyrin enzymes, catalase and peroxidase, have attracted attention in this field. The dependence of catalase formation in the leaf on the iron supply to the plant has been repeatedly demonstrated (1, 5) and the catalase and peroxidase activities of soybean leaves have been shown to be depressed by heavy metal toxicity (30).

Over the past few decades interest has centered on the cytochromes as pathways of electron transfer. Hill and Scarisbrick (16) have devised a method for estimating the total hematin compounds in leaves; Sironval (27) has used this method to show an intimate relationship between these pigments and chlorophyll; other work, especially that concerned with genetical variegation (12, 24), has pointed to a relationship between catalase activity and chlorophyll formation.

Though classical views assign to catalase and peroxidase the function of protecting the organism from the harmful effects of hydrogen peroxide, recent work suggests that catalase and peroxidase play an active part in metabolism, especially so the finding that peroxidase will oxidize a wide variety of biologically occurring compounds in the presence of manganese (2, 13, 18, 19) and that catalase acts as an inhibitor of these reactions. Kamerbeek (17) has also shown that dwarf varieties of plants show much higher peroxidase activities than the corresponding tall varieties, an inverse relationship between growth rate and peroxidase activity being inferred.

Recent studies on the iron nutrition of plants have shown that there is an interdependence among the elements phosphorus, iron, potassium, and calcium (10). Such relationships are strikingly apparent in variegated plants (9) and also in plants suffering from heavy metal toxicity (8). Comparative studies of chlorophyll, catalase, peroxidase, and total heme pigments have accordingly been made in relation to the mineral nutrition of normal mustard plants and of various plants suffering from chlorosis caused by heavy metal toxicity, genetical, or other causes.

METHODS

As described in a previous communication (10), mustard plants (*Sinapis alba*) were grown in nutrient culture containing three levels of iron (Fe_1, Fe_2, Fe_3, resp., 0.1, 0.5, and 2.5 ppm Fe); four ratios of potassium to calcium (K_1, K_2, K_3, K_4, resp. 12/1, 10/3, 7/6, 3/10 meq./1); two ratios of phosphorus to nitrogen (P_1, P_2, resp., 1/11, 2/10 meq/1) such that the sums of the cations and the sums of the anions remained constant. Sodium, magnesium, sulphate, and chloride were supplied in constant amounts; iron was added as the ethylene diamine tetra-acetic acid (EDTA) chelate. These treatments were arranged in a $4 \times 3 \times 2$ factorial design and with two replicates, 48 determinations could be made. When the plants were 3 weeks old, fully expanded upper leaves were taken for determination of catalase and peroxidase activities, chlorophyll and hematin according to methods briefly outlined below. Growth and mineral composition of such plants is discussed elsewhere (10). Other plants were grown or obtained as indicated in the text.

Estimation of chlorophyll was performed according to Comar, Benne, and Buteyn (6). The residue (after extraction of the chlorophyll with acetone which will be termed "acetone residue") was used to determine total hematin contents of the samples. The method of estimating hematin differed from that of Hill and Scarisbrick (16), in that pyridine was added to give the strong absorption of the pyridine hemochrome at 556 mμ, and a photoelectric spectrophotometer (Hilger Uvispek, with glass prism) was used to measure the absorption. Acetone residue (0.1 g) from leaves stripped of midribs was thoroughly ground with 30 mg sodium hydrosulphite in a mortar. This was then mixed with 1.8 ml pyridine/water (2/1 v/v mixture) in the mortar, and the resulting slurry transferred to a 0.5 cm cell. This cell was placed close to the photocell in the spectrophotometer; absorbance readings (A_λ) were made at 570, 555, and 540 mμ. Filter paper was used in place of the reference cell to compensate for light scattering. Its position and thickness were adjusted to give a low absorbance reading at 570 mμ. A slit width of 0.3 mm (equivalent

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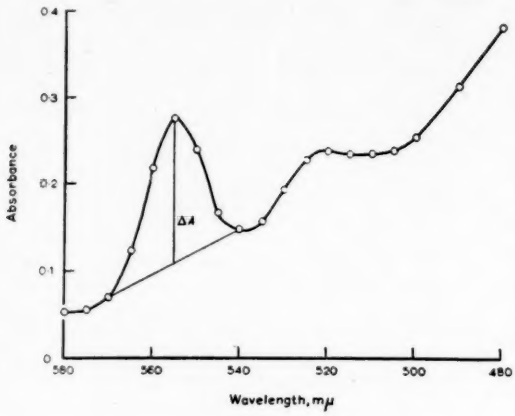

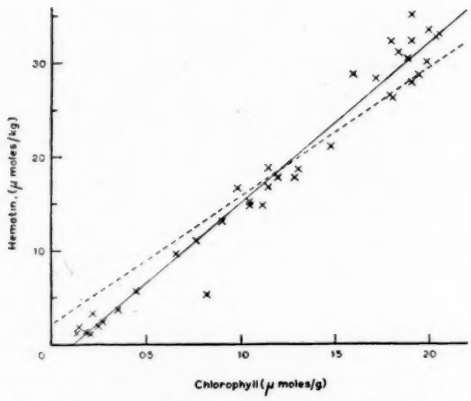

FIG. 1. Absorption spectrum of the pyridine hemochromes in a leaf acetone residue.

FIG. 2. Relationship between hematin and chlorophyll contents of fresh mustard leaves. The full line represents the total regression equation and the dashed line represents the residual regression equation after eliminating differences due to Fe.

to a bandwidth of 1.5 mμ) was used. Figure 1 shows the absorption spectrum of such a slurry, in which the α and β bands of pyridine hemochrome are clearly defined. The height of the α peak above the background, $\triangle A$, is given by $\triangle A = A_{555} - (A_{540} + A_{570})/2$; from this the hematin content was obtained by comparison with a standard. This standard was prepared by adding denatured hemoglobin (4), incorporated in the pyridine/water mixture, to an acetone residue low in hematin. The hemoglobin solution was prepared by lysis of blood cells, and was standardized spectrophotometrically as oxyhemoglobin, for which A^{576}_{mM} was taken as 15.8 (20). The addition of 0.047 μmoles denatured hemoglobin increased the $\triangle A$ (measured on the slurry) from 0.02 to 0.31. From this the concentration of hematin compounds in fresh leaf was calculated as $\triangle A \times 1.62 \times R \times 10^3$ μmoles/kg, where R is the yield of acetone residue from 1 g fresh leaf. Measured $\triangle A$ values ranged from 0.01 to 0.28. Replicate measurements on the same acetone residues indicated a 4.1 % coefficient of variation in $\triangle A$. The accuracy of the hematin estimation is uncertain, as the standard used is not strictly comparable to the samples analyzed. The pyridine hemochrome is uniformly dispersed in the standards, but may be present as insoluble aggregates in the usual acetone residue slurry; if so, our estimates are probably too low by a constant factor (21). Again figure 1 shows that the background scattering does not vary linearly with wave length, as assumed in the calculation: the $\triangle A$ values should probably be about 0.01 units higher than calculated, and the corresponding hematin contents in the fresh leaf should be higher by about two μmoles/kg. In spite of these uncertainties, the method allows a rapid routine comparison of hematin contents in leaves.

Measurement of catalase was made according to the perborate method of Feinstein (15). Leaf tissue (0.5 g) was crushed in a mortar with 5 ml of pH 6.8 phosphate buffer; the macerate then was squeezed through muslin. A series of flasks containing 5 ml of 1.5 % sodium perborate + 1.5 ml phosphate buffer (pH 6.8) was prepared. At zero time 1 ml of macerate was pipetted into each flask; the reaction was stopped in successive flasks after 1, 2, 3, 4, and 5 minutes by rapidly adding 10 ml 2 *N* sulfuric acid. For measurements at zero time the sulfuric acid was added first. The remaining perborate was then titrated with 0.05 *N* potassium permanganate to the first pink color which lasted for 30 seconds. The activity, A, was then calculated according to the formula of Patterson (25) using:

$$r = \frac{4y_4 + 3y_3 - y_2 - 6y_1}{4y_3 + 3y_2 - y_1 - 6y_0} \quad \text{for } t = 0, 1, 2, 3, 4$$

$$\text{or } r = \frac{4y_5 + 4y_4 + 2y_3 - 3y_2 - 7y_1}{4y_4 + 4y_3 + 2y_2 - 3y_1 - 7y_0} \quad \text{for } t = 0, 1, 2, 3, 4, 5$$

where y_0, y_1 . . . . . y_5 are readings at times t_0, t_1 . . . . . t_5 in minutes, $K = -\log_{10} r$ and $A = K/w$ where w is the fresh weight of tissue used, in grams.

Peroxidase activity of leaf tissue was estimated according to the method of Derx (11) as used by Kamerbeek (17), in which 0.5 g of fresh material is thoroughly ground in a porcelain mortar in 96 % alcohol with 0.1 g acid-washed sand and filtered on a 2 inch diameter Buchner funnel with suction using a fine filter paper (Whatman 531). The residue is mixed with 5 ml citrate buffer, left to stand for 1 hour, and filtered as before. Two flasks were then prepared: one contained 1 ml 0.1 *N* H_2O_2, 0.5 ml *o*-toluidine (0.05 %), and 22.5 ml citrate buffer; the other contained 0.5 ml 0.01 *N* ascorbic acid, 4.5 ml

TABLE I

PEROXIDASE VALUES (U.N.P. × 10^4) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF Fe, 4 RATIOS OF K TO Ca, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	44	64	63	58	56	57
K_2	47	61	76	62	61	61
K_3	45	63	56	57	53	55
K_4	33	48	53	46	43	45
		(± 4.9)		(± 4.0)		(± 2.9)
P_1	44	60	62			56
P_2	40	58	62			53
		(± 3.5)				(± 2.0)
Mean	42	59	62			
		(± 2.5)				

Standard errors are given in brackets

citrate buffer and 1 ml of the enzyme solution. After equilibration in a water bath at 25° C, the contents of the two flasks were mixed and the time taken for the formation of the blue color noted. From the time taken, the activity (Unités normale de Peroxidase) is calculated according to the equation U.N.P. $= \frac{150}{a\ t}$ where a is the weight of material in milligrams and t is observed time in seconds.

RESULTS

The statistical treatment of the readings obtained for the peroxidase measurements on leaves of mustard plants grown in a 48 crock experiment are presented in table I. It is quite evident that the peroxidase activity of the mustard leaf is dependent upon the iron supply, since an increase in the iron content of the nutrient medium from 0.1 (Fe_1) to 0.5 ppm (Fe_2) causes a rise of 40 % in the peroxidase value. Further increase of iron to 2.5 ppm (Fe_3) causes only a small increase in the peroxidase value. More remarkable is the apparent relationship between peroxidase activity and the K/Ca ratio of the nutrient medium. The highest K/Ca ratio (K_4) is associated with lowest peroxidase activity.

Catalase activity was dependent only upon the iron level. There was no statistically significant effect of either K/Ca or P/N ratio (table II). But whereas peroxidase showed only a relatively small increase with increase in the iron level, catalase values showed a threefold increase on going from level 1 to 2 of iron, and a fivefold increase on going from level 1 to 3.

The statistical treatment of the chlorophyll and hematin measurements (made on another 48 crock experiment) are presented in tables III and IV. Chlorophyll content increases considerably as the level of iron is raised; fourfold increases were recorded from level 1 to level 2, and eightfold increases from level 1 to level 3 of iron. Apart from a slight depression of chlorophyll content at the highest potassium-calcium ratio (K_4) there was little effect shown by other variants.

TABLE II

CATALASE ACTIVITY (A) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF Fe, 4 RATIOS OF K TO Ca, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	0.43	1.50	1.96	1.40	1.20	1.30
K_2	0.44	1.72	2.11	1.21	1.63	1.42
K_3	0.37	1.31	2.36	1.33	1.36	1.35
K_4	0.52	1.21	2.19	1.23	1.38	1.31
		(± 0.254)		(± 0.207)		(± 0.146)
P_1	0.44	1.51	1.93			1.29
P_2	0.44	1.36	2.38			1.39
		(± 0.179)				(± 0.104)
Mean	0.44	1.44	2.16			
		(± 0.127)				

Standard errors are given in brackets

TABLE III

CHLOROPHYLL (μMOLES/G) CONTENT OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF Fe, 4 RATIOS OF K TO Ca, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	0.30	0.93	1.88	1.11	0.97	1.04
K_2	0.22	1.19	1.90	1.10	1.12	1.11
K_3	0.28	1.16	1.88	1.21	2.13	1.11
K_4	0.17	0.92	1.89	0.91	1.07	0.98
		(± 0.055)		(± 0.045)		(± 0.031)
P_1	0.28	1.03	1.92			1.07
P_2	0.21	1.07	1.85			1.04
		(± 0.039)				(± 0.022)
Mean	0.25	1.05	1.89			
		(± 0.028)				

Standard errors are given in brackets

Since few measurements of hematins were obtained for the lowest iron level, results of only the two highest iron levels could be considered statistically; these (table IV) showed a closely similar pattern to chlorophyll. If the actual measurements of chlorophyll and hematin are compared by the method of least squares, a rectilinear relationship is obtained (fig 2).

Measurements of chlorophyll and hematin were also made on leaves of mustard plants grown in nutrient culture as well as on leaves of sugar beet plants grown in sand culture. They were given nickel (2

TABLE IV

HEMATIN CONTENT (μMOLES/G) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF Fe, 4 RATIOS OF K TO Ca, AND 2 RATIOS OF P TO N

	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	11.5	31.6	23.5	19.7	21.5
K_2	18.0	30.5	24.1	24.5	24.3
K_3	17.1	31.2	25.5	22.8	24.1
K_4	12.7	29.5	18.9	23.3	21.1
	(± 1.49)		(± 1.49)		(± 1.05)
P_1	14.7	31.3			23.0
P_2	15.0	30.2			22.5
	(± 1.05)				(± 0.75)
Mean	14.8	30.7			
	(± 0.75)				

Standard errors are given in brackets

ppm) as the sulphate or EDTA chelate with iron supplied as the chloride or EDTA chelate (8). The results are given in tables V and VI. A similar rectilinear relationship between these two components can be shown for these results, though some aberration occurs at lower values.

Catalase and peroxidase activities of leaves of a similar set of mustard plants grown in the presence of nickel are shown in table V. Since the colorless areas of variegated plants have similar P/Fe and K/Ca ratios to iron deficient plants (9) measurements of total hematin, chlorophyll, catalase, and peroxidase were made on such leaves (table VII).

DISCUSSION

The levels of all four of the cellular components studied showed a clear relationship to the amount of iron supplied to the mustard plants. It is evident, however, that whereas catalase and chlorophyll were practically absent when the iron supply was low, and increased several fold when the iron supply was increased, peroxidase was present in considerable amounts even at the lowest iron level. Peroxidase therefore appears to be an essential component of living matter and is synthesized preferentially when iron supply is inadequate. Peroxidase content also shows an inverse relationship to the potassium-calcium ratio in the nutrient solution.

The similar behavior of catalase and chlorophyll, noted here, has often been observed. This probably implies closely associated pathways of biosynthesis, as is clearly shown in the results of Mikhlin and Mutuskin (23). They find an inverse relationship between peroxidase on the one hand and catalase and chlorophyll on the other. Schwarze (26) has argued against an inverse relationship between catalase and peroxidase in plant tissue. Inspection of his results shows, however, that though there may be little dif-

TABLE V

CHLOROPHYLL AND HEMATIN, CATALASE (A) AND PEROXIDASE (U.N.P. × 10^4) IN MUSTARD LEAVES GROWN IN PRESENCE OF NICKEL (2 PPM) AS SULPHATE OR EDTA CHELATE (NiV) WITH IRON (2 PPM) ADDED AS CHLORIDE OR EDTA CHELATE (FeV)

TREATMENT	% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH	CATALASE (A)	PEROXIDASE (U.N.P. × 10^4)
1. $NiSO_4$ + $FeCl_3$	12.4	11.4	0.38	0.755	209
2. $NiSO_4$ + FeV	13.1	20.9	0.79	1.945	399
3. NiV + $FeCl_3$	10.7	36.8	1.81	0.525	72
4. NiV + FeV	10.2	36.8	1.88	1.975	102
5. $FeCl_3$ Control	7.0	12.3	0.66	0.655	143
6. FeV Control	9.3	36.8	2.21	2.500	73
Standard error	...	...	...	± 0.089	± 22

TABLE VI

CHLOROPHYLL AND HEMATIN IN FRESH LEAVES OF NORMAL (A) AND NICKEL-TOXIC (B) SUGAR BEET LEAVES

TREATMENT	% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH
A Healthy green	8.4	20.5	1.49
A " "	10.5	24.1	1.47
B Slight chlorosis	10.7	15.9	0.79
B Intermediate chlorosis	8.3	12.3	0.63
B Severe chlorosis	9.7	6.8	0.00

TABLE VII

CHLOROPHYLL, HEMATIN, CATALASE (A) AND PEROXIDASE (U.N.P. × 10^4) OF GREEN (G) AND ALBINO (W) PORTIONS OF VARIEGATED LEAVES OF VARIOUS PLANTS

LEAF		% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH	CATALASE	PEROXIDASE
Bougainvillea glabra	G	17.4	36.4	1.88	2.28	61.2
" "	W	10.0	10.0	0.08	0.22	75.0
Sambucus nigra	G	13.7	24.1	1.90	2.90	42.9
" "	W	9.5	11.4	0.03	0.69	52.1
Hosta fortunei	G	...	...	...	4.24	50.0
" "	W	...	...	...	0.32	64.0
Chlorophytum comosum	G	9.8	16.9	1.12	1.60	25.0
" "	W	6.1	3.2	0.03	0.58	73.0
Spiraea japonica	G	20.2	23.8	1.17	1.10	27.8
" "	W	14.8	8.8	0.01	0.55	104.0

ference between the peroxidase activity of green and chlorotic tissue, catalase is always considerably lower in chlorotic tissue, whether chlorosis is due to nutrient deficiency, genetical causes, or even darkness. Our results show that irrespective of the cause of chlorosis, the peroxidase-catalase ratio is always higher in plants showing an iron-deficiency type of chlorosis. Such a relative relationship could also explain apparent anomalies of catalase and peroxidase activity reported by Appleman (3) and Woods and Dubuy (31).

Sironval (28) has found no simple relationship between peroxidase, catalase, and chlorophyll. He noticed, however, that the activities of catalase and peroxidase at different stages in development of the leaf "thus vary in an inverse manner, as if they rearrange themselves during the development of the leaf", conclusions conforming with those found here.

Lundegardh (22) has discussed the possibility of peroxidase playing a role in terminal oxidation of the cell. He mentioned its insensitivity to inhibitors such as azide and carbon monoxide. His calculations suggest that respiration due to peroxidase proceeds at so low an intensity as to make little difference to the cyanide-sensitive respiration. He has noted, however, that high concentrations of oxygen reduce the inhibiting effect of cyanide on respiration; it may well be that the in vivo hydrogen peroxide concentration is so low that peroxidase behaves differently from in vitro experiments where the hydrogen peroxide concentration is high. The presence of catalase in the living cell ensures a low peroxide concentration; Kenten and Mann (19) have found catalase to be inhibitory to the peroxidase-manganese oxidizing system. Some participation of peroxidase in terminal oxidation cannot, therefore, be excluded.

Manganese is connected intimately with iron metabolism; in the plant their relative amounts are of importance (29). Excess of manganese causes an iron-deficiency chlorosis while a deficiency of manganese causes an iron toxicity chlorosis (8). It would appear then that manganese plays an important part in oxidation. Apart from the studies of Kenten and Mann (19), however, no mechanism of oxidative metabolism involving manganese has been suggested.

The rectilinear relationship between chlorophyll and total hematin (full line in fig 2) has been reported by Sironval (27) and by Davenport (7). In agreement with these workers, we find the ratio of chlorophyll to hematin molecules to be about sixty to one. The relationship can be represented by the total regression equation:

$$y = 17.05x - 1.84.$$

where y represents the hematin and x the chlorophyll values. This appears to show that hematin declines to zero before chlorophyll disappears entirely. This may be due to the approximation used in correcting for background scattering as indicated under methods. An analysis of covariance was carried out in which Fe was the only factor of classification considered. This showed that differences in hematin due to the level of iron are greater than can be accounted for by the differences in chlorophyll. The regression for the relationship within the iron levels is less steep and given by $y = 13.78x + 2.16$ (shown by a dashed line in fig 2). Thus a positive hematin value is indicated when chlorophyll is zero.

In any case it is clear that little hematin remains at low chlorophyll levels when chlorosis is due to iron deficiency. This is in contrast to the results obtained with chlorotic tissue from variegated leaves (table VII), and with leaves of mustard (table V) and sugar beet (table VI) in which chlorosis is due to nickel toxicity. Here significant amounts of hematin compounds remain even when chlorophyll is almost absent, although the hematin levels are always much lower in chlorotic than in corresponding green and healthy tissue, in accordance with earlier findings (9).

Summary

A study was made of the catalase and peroxidase activities as well as of chlorophyll and total hematin contents of mustard plants. The plants were grown in nutrient culture with different iron, potassium, calcium, phosphorus, and nitrogen levels. Leaves of nickel-toxic and variegated plants were also studied. Peroxidase activity showed only small variations between chlorotic and green leaves, whereas catalase activity was several fold greater in green than in chlorotic leaves. A linear relationship of 60:1 between chlorophyll and hematin was found.

A spectrophotometric method is reported for determining total hematin compounds as pyridine hemochrome in acetone residues derived from leaves.

Acknowledgment

We wish to thank Dr. H. E. Davenport for a useful discussion of the method of estimating hematin.

Literature Cited

1. Abadia, A. 1956. Chlorophyll synthesis in induced iron deficiency. Ann. estac. exp. Aula Dei 4: 212–261.
2. Akazawa, T. and E. C. Conn. 1958. Oxidation of reduced pyridine nucleotides by peroxidase. Jour. biol. Chem. 232: 403–415.
3. Appleman, D. 1952. Catalase-chlorophyll relationship in barley seedlings. Plant Physiol. 27: 613–620.
4. Baldwin, E. and D. J. Bell. 1955. Cole's Practical Physiological Chemistry. P. 164. Heffer & Sons, Ltd., Cambridge, England.
5. Bannerjee, S. 1957. Catalase activity of soybeans grown at various concentrations of iron. Jour. Indian Soc. Soil Sci. 5: 169–172.
6. Comar, C. L., E. J. Benne, and E. K. Buteyn. 1943. Calibration of a photoelectric colorimeter for the determination of chlorophyll. Ind. Eng. Chem. Anal. Ed. 15: 524–526.
7. Davenport, H. E. 1958. The effects of some micronutrient deficiencies on the concentration of haem and chlorophyll in leaves. Proc. IV Int. Congr. Biochem., Vienna 146.
8. Dekock, P. C. 1956. Heavy metal toxicity and iron chlorosis. Ann. Bot. 22: 133–141.
9. DeKock, P. C. and A. Hall. 1955. The phosphorus-iron relationship in genetical chlorosis. Plant Physiol. 30: 293–295.
10. Dekock, P. C., A. Hall, and Margaret McDonald. 1960. A correlation between the ratio of phosphorus to iron and potassium to calcium in mustard leaves. Plant & Soil XII: 128–142.
11. Derx, H. G. 1942. Exposé d'une methode chronométrique pour le dosage de la peroxydase. Proc. Acad. Sci. Amsterdam 45: 718–725.
12. Von Euler, H., U. Gard, and G. Rislund. 1931. Katalase und Zucker bestimmungen in chlorophylldefekten Pflanzen. Zeits. physiol. chem. 203: 165–177.
13. Galston, A. W., J. Bonner, and R. S. Baker. 1953. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. Arch. Biochem. 42: 456–470.
14. Granick, S. and L. Bogorad. 1952. The biosynthetic chain of heme and chlorophyll. II Int. Cong. Biochim. Paris. 323–324.
15. Feinstein, R. M. 1949. Perborate as a substrate in a new assay of catalase. Jour. biol. Chem. 180: 1197–1202.
16. Hill, R. and R. Scarisbrick. 1951. The hematin compounds of leaves. New Phytol. 50: 98–111.
17. Kamerbeek, G. A. 1956. Peroxidase content of dwarf types and giant types of plants. Acta Bot. Néerl. 5: 257–263.
18. Kenten, R. H. 1955. The oxidation of γ-(3-Indolyl) propionic acid and γ-(3-Indolyl) propionic acid and γ-(3-Indolyl)-*n*-butyric acid by peroxidase and Mn^{2+}. Biochem. Jour. 61: 353–359.
19. Kenten, R. H. and P. J. G. Mann. 1950. The oxidation of manganese by peroxidase systems. Biochem. Jour. 46: 67–73.
20. Lemberg, R. and J. W. Legge. 1949. Hematin Compounds and Bile Pigments. Interscience Publishers, Inc., New York.
21. Lothian, G. F. and P. C. Lewis. 1956. Spectrophotometry of granulated materials, with particular reference to blood corpuscles. Nature 178: 1342–1343.
22. Lundegardh, H. 1954. Enzyme systems conducting the aerobic respiration of roots of wheat and rye. Arkiv. Kemi. 7: 451–478.
23. Mikhlin, D. M. and A. A. Mutuskin. 1956. Distribution of the iron-porphyrin enzymes among different parts of barley plants. Biochemistry, Leningrad 21: 133–142.
24. Nakamura, V. H. 1941. Uber die quantitativen Beziehungen zwischen der Katalase in Chloroplasten und dem Chlorophyll, nebst einigen Bemerkungen uber die Rolle der Katalase in Assimilationsvorgang. Jap. Jour. Bot. 11: 221–236.
25. Patterson, H. D. 1956. A simple method for fitting an asymptotic regression curve. Biometrics 12: 323–329.
26. Schwarze, P. 1954. Beziehungen Swischen Peroxydasereaktion, Eiwiss-Spiegel, und Chlorophyllbildung. Planta 44: 491–502.
27. Sironval, C. 1958. Day-length and hematin compounds in plants. Nature, London 182: 1170–1171.
28. Sironval, C. 1959. Sur l'activite catalasique et peroxidasique des feuilles. Arch. intern. Physiol. Biochim. 67: 125–126.
29. Somers, I. I. and J. W. Shive. 1942. The iron-manganese ratio in relation to plant metabolism. Plant Physiol. 17: 582–602.
30. Wallace, J. M. 1957. The influence of high nutrient levels of Co, Cu, and Mn on leaf catalase and root peroxidase activity in soybean plants grown with different levels of iron. Dissertation Abstr. XVII: 2398.
31. Woods, M. W. and H. G. Dubuy. 1950. Oxidising enzymes in variegated leaves and in isolated normal and mutant plastids. Amer. Jour. Bot. 33: 828.

SOME OBSERVATIONS ON ABSORPTION OF CESIUM BY EXCISED BARLEY ROOTS[1, 2]

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INTRODUCTION

Because Cs 137 is one of the major long-lived products of nuclear fission, there is a growing interest in the uptake of cesium by plants and its subsequent introduction into food chains. At the same time, the fact that Cs is readily absorbed by plants from culture solutions makes it useful in studies of the general aspects of ion uptake. Its use has the additional advantage that the initial cesium content of the tissue is essentially zero.

The purpose of the following study was to establish some fundamental relationships in Cs absorption such as the influence of external Cs concentration, the influence of low temperature and anoxybiosis, and the interaction with other mono- and divalent cations.

MATERIAL AND METHODS

Excised root material from Tennessee Winter barley, 1956 crop, prepared as described by Jacobson et al (1950), was used for these experiments.

Unless otherwise stated, 1 g (fresh wt) of root material in 3.5 liters of solution was used. To avoid absorption of Cs by glass at low concentrations, practically all experiments were performed in polyethylene bottles. During the absorption period the solutions were aerated with compressed air from the laboratory supply and kept at 25° C by immersion in a waterbath. The initial pH of all solutions was 5.5. In a few cases it was necessary to add some alkali for maintaining pH during the experiment; this caused negligible changes in the cation concentration(s).

At the end of an experiment the roots were separated from the solutions by means of a nylon mesh filter, washed with distilled water for about ten seconds, dried in a thermostat at 105° C, and ashed at 550° C in a period of 20 minutes. The ash was digested with $\sim 1\,N$ HCl on a steambath for about 20 minutes and filtered. The filtrate was evaporated to dryness on a steambath and the residue was redissolved in 10 ml of dilute HCl. In this way sufficiently high Cs concentrations were obtained to allow accurate determination with the Beckman flame photometer (model DU). The wavelength used was 852 mμ and the slit width was 0.03 mm throughout. The calibration graph used for interpolation was derived from eight standard solutions ranging from 0.1 to 0.8 meq/liter and appeared to be linear in this range.

Since steady state rates of Cs uptake were to be determined, it was important to exclude rapid initial equilibrations. This initial stage appeared to be brief (5 min or less) both at 5 meq/l and at 0.01 meq/l Cs (see fig 1). Therefore where steady state rates were derived, the experiments were carried out using two absorption periods, one 3 or 1½ hours and the other ½ hour, and the steady state uptake for 2½ or 1 hour, respectively, was obtained by subtraction. At low rates of Cs uptake this procedure sometimes led to slightly negative values for Cs absorption (cf. fig 4A).

Because at very low Cs concentrations there is still a high rate of Cs uptake, in a number of cases the roots had to be transferred to fresh solutions one or two times during the experiment to avoid an appreciable decrease in the concentration of the culture solution.

Experiments at low temperature (1° C) were run in a cold room. Where anoxybiosis was required, N_2 was bubbled through the solutions for about 16 hours before as well as during the experiment. To remove the last traces of O_2, the N_2 stream was led over glowing red copper coils.

All cations used were added as their chlorides. The CsCl used had the following composition: CsCl 98.95 %, RbCl 0.74 %, KCl 0.04 %, NaCl 0.20 %, moisture 0.03 %.

The results were calculated on a kg fresh weight basis. Unless otherwise stated all values in the graphs represent single samples.

RESULTS

In the range of low concentrations (from 0.0 to 0.5 meq/l) the rate of Cs absorption asymtotically approaches a constant value as the external Cs concentration is increased. The half-value concentration is about 0.008 meq/l (see fig 2A). However, this behavior in the range of a few tenths of a meq/l appears to be only a matter of the interval of concentration considered. Experiments at higher concentrations (up to 75 meq/l) disclosed a further rise in the rate of Cs uptake with concentration (see fig 2B).

The influence of low temperature and anoxybiosis on Cs absorption was studied at a concentration of 0.1 and of 5 meq/l. At an external Cs concentration of 5 meq/l a considerable rate of Cs absorption was observed both at 1° C and in a N_2 atmosphere (see fig 3A). At the same time there was a considerable loss of internal K from the tissue during anoxybiosis (see fig 3B).

At a concentration of 0.1 meq/l Cs absorption was completely checked, however, by O_2 exclusion after the 1st hour (fig 3B). The low rate of uptake during the 1st hour may have been due to the introduc-

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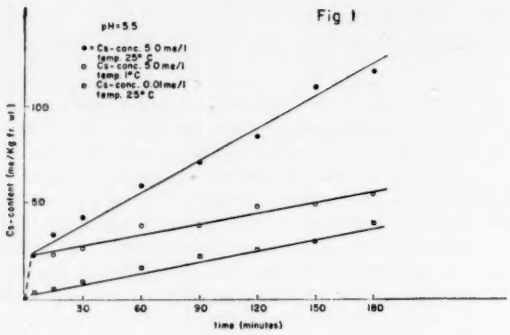
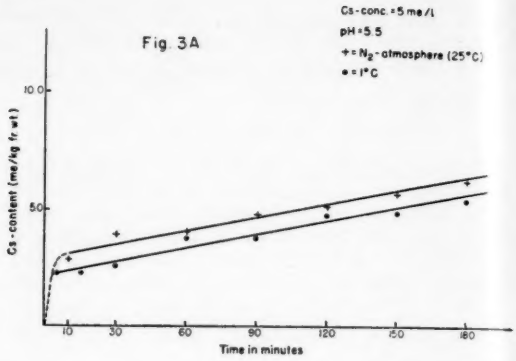
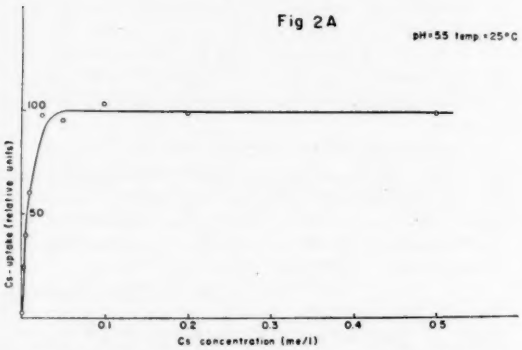






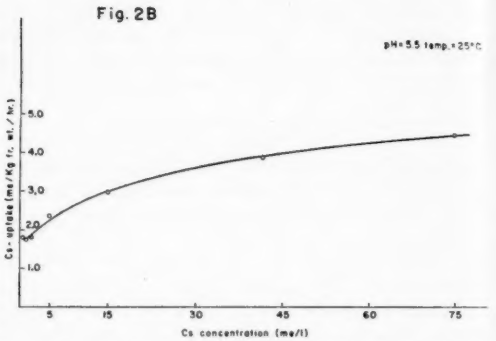


FIG. 1. Cesium content of barley roots as affected by time of immersion in 5 meq/l CsCl solutions at 1° C and 25° C and in 0.1 meq/l CsCl solutions at 25° C.

FIG. 2A. Cesium absorption by barley roots from CsCl solutions of varying concentration. On the ordinate, 100 relative units correspond to about 2.0 meq Cs per 1,000 g fresh wt/hour.

FIG. 2B. Cesium absorption by barley roots from CsCl solutions of varying concentration. The Cs uptake is expressed as meq per 1,000 g fresh wt/hour. Values represent duplicate samples.

FIG. 3A. Cesium content of barley roots as influenced by time of immersion in 5 meq/l CsCl solutions 1) at 1° C in air and 2) at 25° C in an atmosphere of N_2.

tion of a small amount of oxygen into the bottles with the roots.

In the other experiments, the interference by other mono- and divalent cations with Cs absorption in the range of low concentrations (from 0.0 to 0.1 meq/l) was studied. The concentration of the interfering ions was 0.1 meq/l throughout. As the capacity for Cs absorption differs somewhat for different sets of roots, a special experiment was performed in order to put the interference curves on a comparable basis. In this experiment one set of roots was used to compare Cs absorption from solutions without and with 0.1 meq/l of the interfering ions. The ratios computed were used in constructing figures 4A and 4B. This procedure is based on the assumption that although the magnitude of the Cs absorption varies somewhat from one set of roots to another, the shape of the absorption isotherm is not altered.

There appeared to be a strong inhibition of Cs uptake in the presence of K and, to a somewhat less extent, in the presence of Rb (see fig 4A). Also NH_4 had a weak inhibiting effect on Cs absorption. In all three cases there was a clear change in the general shape of the absorption curve.

If Na, Li, Ca, or Mg was added, however, the shape of the absorption curve was unaffected but in some cases the overall absorption capacity changed (see fig 4B). It was somewhat lower in the presence of Na and somewhat higher in the presence of Ca. Li did not affect it, and the effect of Mg was within the experimental error.

As for the simultaneous absorption of the interfering ion (see fig 4C), Rb absorption was hardly affected by the presence of increasing amounts of Cs whereas a considerable decrease in Na absorption was observed under the same conditions. Li was hardly taken up at all at a concentration of 0.1 meq/l and its absorption was unaffected by the presence of Cs. As for K, the initial K content of the tissue was high ($\sim$ 10 meq/kg fr. wt) causing a considerable variation in the K data. K absorption averaged 1.7 meq/kg fr. wt/hr at a concentration of 0.1 meq/l but whether it was affected by the presence of Cs or not, could not

be established. The absorption of the other ions was not determined.

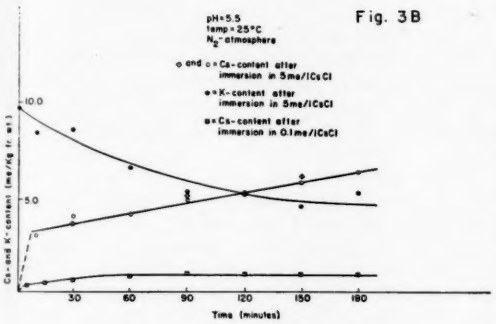
Discussion

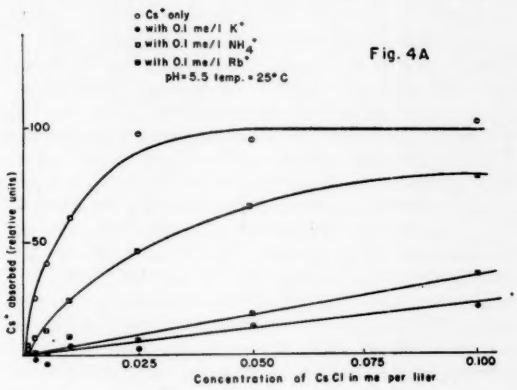
It is tempting to explain the nature of the relationship between Cs absorption and external Cs concentration on the basis of the hypothesis that two mechanisms are involved in Cs uptake. On this assumption a very effective absorption mechanism is working at its maximal capacity at a concentration as low as 0.1 meq/l whereas a second but far less effective absorption system becomes important only at concentrations a hundred-fold higher.

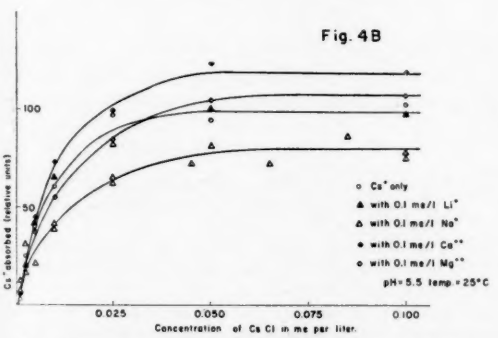
This hypothesis is supported by the results obtained at low temperature and in a N_2 atmosphere. Again the most logical interpretation seems to be that there are two absorption mechanisms, one at work at low concentrations and sensitive to conditions checking normal respiration and the other coming into play at much higher concentrations only and insensitive to anoxybiosis.

The foregoing assumption does not necessarily imply that the algebraic sum of the saturation level of the most effective absorption mechanism and the rate of Cs uptake at 5 meq/l under conditions of anoxybiosis or low temperature equals the rate of Cs absorption at 5 meq/l under normal conditions. The two systems may not work quite independently.

A most obvious conclusion from the available data would be that the second mechanism is passive and consists of a slow exchange for internal ions such as K. This view is supported by the fact that in some preliminary experiments a loss of internal K was found after 3 hours immersion of the roots in a







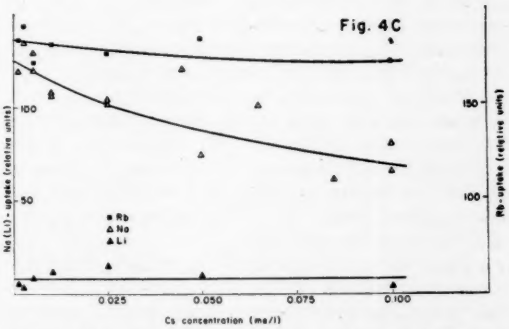


Fig. 3B. Cesium content of barley roots as influenced by time of immersion in 0.1 and 5 meq/l CsCl solutions in an atmosphere of N_2. Potassium content of barley roots as a function of time of immersion in 5 meq/l CsCl solutions in an atmosphere of N_2.

Fig. 4A. The effect of the presence of 0.1 meq/l K^+, NH_4^+, and Rb^+ on the absorption of Cs^+ by barley roots from CsCl solutions of varying concentration. On the ordinate, 100 relative units correspond to about 2.0 meq Cs^+ per 1,000 g fresh wt/hour.

Fig. 4B. The effect of the presence of 0.1 meq/l Li^+, Na^+, Ca^{++}, and Mg^{++} on the absorption of Cs^+ by barley roots from CsCl solutions of varying concentration. On the ordinate, 100 relative units correspond to about 2.0 meq Cs^+ per 1,000 g fresh wt/hour.

Fig. 4C. The uptake of Na^+, Li^+, Rb^+ by barley roots from CsCl solutions of varying Cs^+ concentration but containing Na^+, Li^+, or Rb^+ at a concentration of 0.1 meq/l. On the ordinates, 100 relative units correspond to about 2.9 meq per 1,000 g fresh wt/hour for the absorption of Na^+ and Li^+ and to about 2.3 meq per 1,000 g fresh wt/hour for the absorption of Rb^+.

solution of 5 meq/l Cs whereas no comparable loss was found after 3 hours immersion in distilled water. However, we cannot explain on the basis of this assumption why the still quite considerable rate of Cs absorption (~ 1 meq/kg fr. wt/hr) at low temperature and during anoxybiosis did not decrease with time as did the rate of K loss. A practically constant rate of exchange would require a much larger bulk of exchangeable ions than can be actually present in this tissue.

The specificity of the mechanism responsible for Cs absorption at low concentrations appeared to be small. In fact it is obvious that Cs will be transported by a mechanism transporting one or more other ion-species in a normal nutritional environment. It is most likely that Cs makes use of the mechanism transporting K since both K and Rb strongly inhibited Cs uptake in a way to be expected in the case of a direct competition for the same binding site. This conclusion is supported by the work of Collander (1937) who found that K, Rb, and Cs are absorbed at about the same rate by a variety of plants. Epstein and Hagen (1952) conclude likewise that K, Rb, and Cs are bound by the same reactive centers in barley. Their experiments were performed at much higher concentrations (1–10 meq/l), however, so that a comparison with their results might be rightly questioned.

The affinity of the binding sites was higher for K and Rb than for Cs since in the presence of an equivalent amount of K or Rb, Cs absorption was reduced by about 75 % and 65 % respectively (cf. fig 4A). The rate of uptake from a 0.1 meq/l solution was much lower for Cs (~ 2.0 meq/kg fr. wt/hr) than for Rb (~ 4.4 meq/kg fr. wt/hr) but higher than for K (~ 1.7 meq/kg fr. wt/hr). That is, the same mechanism may transport one ion-species at a higher rate than another (cf. Bange 1959). The relatively low rate of K absorption may be connected with the relatively high initial K content of the tissue.

A similar preference for K as compared to Cs is apparent from the work of Menzel and Heald (1955). The low value (0.2) of their distribution factor (=Cs/K ratio in the plant: Cs/K ratio in the solution) for a number of plants may be due either to a lower affinity for Cs or to a lower rate of transport of Cs by the same mechanism.

The fact that Na affected the overall absorption capacity for Cs only and not the half-value concentration of the absorption curve provides indirect evidence that K and Na are transported by different mechanisms in barley. Epstein and Hagen (1952) draw the same conclusion but again their statement applies to much higher concentrations.

It was somewhat surprising, however, that in the experiments with Rb the rate of Rb absorption did not decrease as the rate of Cs absorption increased with rising Cs concentration (fig 4C). A similar observation is mentioned by Sutcliffe (1956, 1957). This phenomenon certainly needs further attention because it conflicts with the usually applied competition kinetics. It is likewise difficult to explain the decrease in the rate of Na absorption with increasing Cs concentration (fig 4C). The initial Na content of the tissue was about one meq/kg fr. wt.

The NH_4 ion also appears to show affinity for the same site as Cs although it competes far less than K and Rb. Interactions in absorption between NH_4 and other cations have not yet been studied in detail as far as the authors are aware.

Ca showed the stimulating effect on Cs absorption known as the Viets effect. The relative stimulation appeared to be independent of the rate of Cs absorption. The nature of the Viets effect is still obscure.

Summary

Steady state Cs absorption was studied in relation to the external Cs concentration and under conditions of low temperature and anoxybiosis. The results are tentatively explained on the basis of a two-fold absorption mechanism. The first mechanism is very effective, reaching a saturation level at a concentration as low as 0.1 meq/l (half-value ~ 0.008 meq/l). Absorption at this concentration was shown to be sensitive to anoxybiosis. The second mechanism becomes relatively important only at concentrations a hundredfold higher and is insensitive to anoxybiosis. The possibility of a passive nature of the second mechanism is discussed.

The interference by other mono- and divalent cations with Cs absorption in the concentration range from 0.0 to 0.1 meq/l was studied. K, Rb, and to a less extent NH_4 inhibited Cs uptake in a way suggesting competition for the same binding site. Li and Mg had little effect on Cs absorption; Na and Ca only affected the overall absorption capacity for Cs; Na in a negative and Ca in a positive sense.

Acknowledgments

The authors are greatly indebted to Mr. H. M. King for technical assistance with the experiments.

Literature Cited

1. Bange, G. G. J. 1959. Interactions in the potassium and sodium absorption by intact maize seedlings. Plant and Soil. 11: 17–29.
2. Collander, R. 1937. Ueber die Kationenelektion der höheren Pflanzen. Ber. deut. bot. Ges. 55: 74–81.
3. Epstein, E. and C. E. Hagen. 1952. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–475.
4. Jacobson, L., R. Overstreet, H. M. King, and R. Handley. 1950. A study of potassium absorption by barley roots. Plant Physiol. 25: 639–647.
5. Menzel, R. G. and W. R. Heald. 1955. Distribution of K, Rb, Cs, Ca, and Sr within plants grown in nutrient solutions. Soil Sci. 80: 287–293.
6. Sutcliffe, J. F. 1956. The selective absorption of alkali cations by storage tissues and intact barley plants. Potassium Symposium: Vol.: 1–11.
7. Sutcliffe, J. F. 1957. The selective uptake of alkali cations by red beet root tissue. Jour. Exp. Bot. 8: 36–50.

SIMPLE METHOD FOR CONTINUOUS TREATMENT OF PLANT MATERIAL WITH METERED TRACES OF ETHYLENE OR OTHER GASES[1,2]

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For investigating the effects of ethylene gas on plant material (e.g., fruit ripening or morphological responses) known concentrations in the test atmosphere of less than 1,000 parts per million (0.1 %) are usually wanted. These atmospheres have been variously obtained. Some workers have used ripe fruit or cultures of *Penicillium digitatum* Sacc. as sources of a low (but unknown) concentration of ethylene. Others have used flowmeter combinations, bubble counting systems, or pressure cylinders made up to specified concentrations. In closed systems, low known concentrations of ethylene may be achieved by pipetting small amounts of gas into the system or (for very low concentrations) by pipetting portions of ethylene-saturated water or dilutions thereof (8).

For systems in which a continuous flow of the test atmosphere is desired, accurate regulation of the trace component is difficult. Combinations of capillary flowmeters, such as have been used for modified atmosphere studies (1, 2), are not sufficiently sensitive for regulation of the trace component, although quite satisfactory for major constituents of a mixture. (Capillary flowmeters are to be preferred over rotameters, because they can be made cheaply in any laboratory for any flow rate; they also give more stable readings). If a Mariotte bottle is used to regulate the flow of the trace constituent, the desired accuracy can be achieved in a system that will remain stable and can be calibrated and adjusted easily. This method was used and described by Huelin (6), but when his thesis was published it was not described (7). Griffiths and Potter refer briefly to the technique (5).

The Mariotte bottle (4, 11) should be better known to plant physiologists. Its theory has been described by Schwertz (12) in an application to flowmeter calibration, and applications in post-harvest physiology have been shown by Platenius (9) and Pratt (10). The Mariotte bottle will maintain a constant very small flow of liquid under a constant pressure; hence, it can be used to displace a small flow of gas from a reservoir through a flowmeter into a larger air flow. Adjustments of the relative rates of flow of the trace gas and the air make a wide range of concentrations readily available. Huelin's basic method (6) is described in this note with the inclusion of modifications which extend its range of usefulness. In our laboratory, the method has been used to maintain concentrations ranging from 0.003 to 1000 ppm.

MATERIALS

Figure 1 presents the basic method with its useful modifications. The volume of air required for a particular plant sample or group of samples is provided from a supply of pure compressed air which is humidified (H) and metered through a calibrated flowmeter (J). Stable and accurate regulation of this main air stream, over a wide range of flow rates, is accomplished by use of a barostat tower (I). Note that many air supplies are not pure enough for critical investigations or for making reliable concentrations of ethylene in air of the order of one part per million or lower. Air compressor intakes are often located where they may pick up automobile exhaust or other fumes. Hence, for investigating the physiological effects of ethylene, the purity of the control air supply must be investigated.

The central part of figure 1 shows how a Mariotte bottle regulates the addition of the desired trace component to the above air supply through a fine capillary flowmeter which is joined to the main system through a tee. The Mariotte bottle (A) provides the regulation of a steady flow (dropwise) of liquid into the gas reservoir (C). The effective pressure (h) is independent of the volume of liquid in (A) and is adjusted by raising or lowering either the capillary air-inlet tube (B) or the entire Mariotte bottle. (The connection B-G is not needed in a simple system without back-pressure). The liquid displaces gas from the reservoir (C) through the capillary (D), and the rate of gas flow is proportional to the pressure indicated by the manometer (E). The gas then flows into the metered air stream at the tee (F).

The volumes of the Mariotte bottle and gas reservoir are selected in relation to the volume of gas to be dispensed between refills. For instance, to obtain a concentration of 1,000 ppm, 20 ml of ethylene per hour can conveniently be added to an airflow of 20 liters per hour, using a 500 ml graduated cylinder as the gas reservoir and a 4 liter Mariotte bottle; renewal will be required only once a day. For the same concentration in an air flow of 200 liters per

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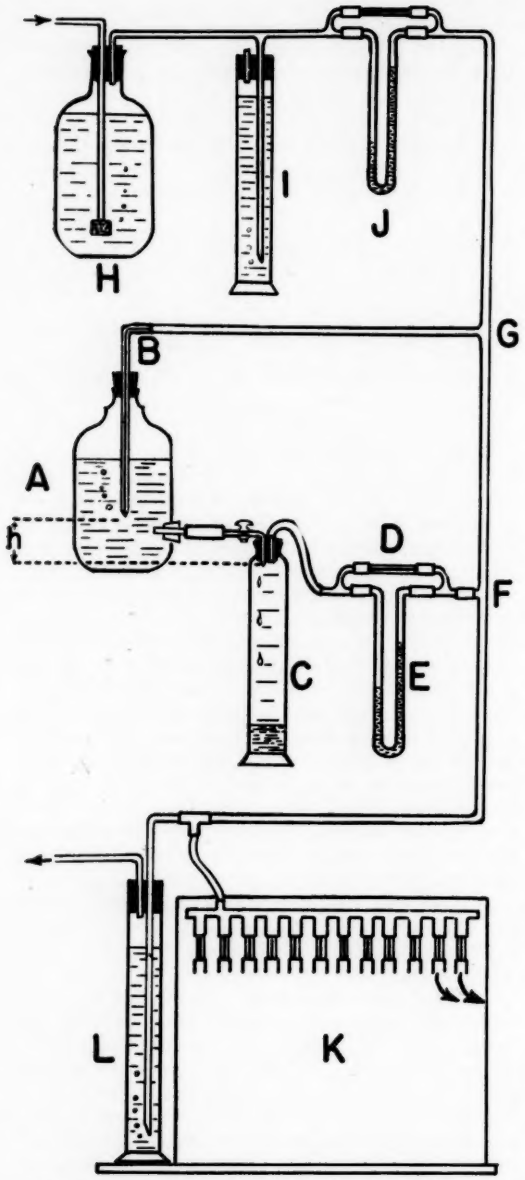

FIG. 1. Apparatus for the continuous metering of traces of gas into a measured air stream, with provision for further division of the gas mixture to separate samples.

hour, proportionally larger reservoirs and Mariotte bottles are desirable.

This apparatus may be calibrated by observation of either the volume of displacing liquid or the volume of gas delivered. It is best to calibrate it under the conditions of use by observation of the volume of displacing liquid in the gas reservoir at convenient intervals of time. This also provides a continuous check on accuracy during operation. The gas being metered must be essentially insoluble in the displacing liquid; otherwise, the volume of liquid flowing into the gas reservoir will be greater than the volume of gas flowing out of the reservoir into the system. As Huelin (6) found, ethylene is sufficiently soluble in water to make this difference appreciable; he applied a correction, while Griffiths and Potter (5) used ethylene-saturated water in the Mariotte bottle. It is more convenient and more accurate to salt out the ethylene; for example the use of 20 % ammonium sulfate solution as the displacing fluid reduces the solubility of ethylene to insignificance. For other gases, the displacing solution might be made acid or alkaline, or another fluid could be used.

By varying one or more of the following, a wide range of gas dilutions may readily be obtained: (1st) The pressure difference across the capillary (D) may be varied within the limits of the experimental apparatus; (2nd) the length and bore of the capillary (D) may be varied; (3rd) the gas in the reservoir (C) may be any conveniently prepared dilution, and (4th) the rate of the air flow with which the gas is mixed (at F) may be varied. The viscosity of other gases may be appreciably different from that of air, so the capillary (D) must be calibrated for the particular gas or mixture used in the reservoir (C). As a first approximation, the viscosity of ethylene is about half that of air, and a capillary previously calibrated for air can be corrected on this assumption. Figure 2 illustrates the uniformity with which the desired gas may be added to an air stream and the effect of gas viscosity on the rate of flow. In this instance, the gas was metered into an air flow of 190 liters per hour; however, essentially the same results were obtained when the gas was passed into the open atmosphere. The method will work satisfactorily only at constant temperature. If the ambient temperature fluctuates, the pressure in the gas reservoir

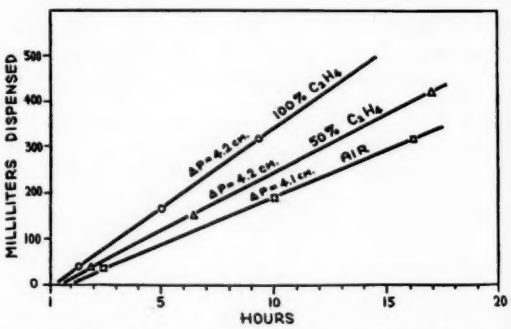

FIG. 2. Volume of trace component (ethylene) metered from the gas reservoir (fig 1, C) after indicated times. Note the uniformity of flow rate and the effect of gas composition. Pressures ($\triangle$ P) are in centimeters of water.

will vary and uneven flows result. Another hazard of fluctuating temperature is condensation of water in the capillary flowmeter; for accuracy, the capillary must remain clean and dry.

If any back-pressure exists in the system at (F), due to attachment of further flow controls (fig 1, K), absorbing towers, etc., the simple system described thus far will not work. The Mariotte bottle dispensing system and the main air stream are usually operated at different head pressures, so that a back pressure at (F) will affect the two gas flows disproportionately. In fact, the air pressure is usually so great that any back pressure will cause the Mariotte system (A–F) to be blown out by a strong back flow. If the connection (B–G) is made to the Mariotte bottle air inlet, this difficulty is eliminated.

When the same gas mixture is to be used on several samples of tissue, the complete system shown in figure 1 allows one Mariotte bottle and trace gas supply to serve the entire system. An air supply slightly larger than required for all samples is humidified by the water bubbler (H), measured by the flowmeter (J), and regulated by the barostat tower (I). To this known air supply, ethylene (or other gas) is added as already described. After leaving the mixing tee (F), the dilute gas mixture enters a manifold and flowmeter board (K) of the sort described by Claypool and Keefer (3). Each individual sample is supplied a known total air-ethylene flow which is measured by a calibrated capillary, and these flows are regulated by the barostat (L). The small excess flow (from L) and the flows from all samples should be exhausted to the outside to minimize the ethylene contamination of the storage room.

Summary

Traces of ethylene or other gases can be metered accurately into larger air streams by the use of a Mariotte bottle and simple combinations of flowmeters. Special applications are described.

Literature Cited

1. Biale, J. B. 1946. Effect of oxygen concentration on respiration of the Fuerte avocado fruit. Amer. Jour. Bot. 33: 363–373.
2. Claypool, L. L., and F. W. Allen. 1947. Modified atmospheres in relation to the transportation of deciduous fruits. Proc. Amer. Soc. Hort. Sci. 49: 92–98.
3. Claypool, L. L., and R. M. Keefer. 1942. A colorimetric method for CO_2 determination in respiration studies. Proc. Amer. Soc. Hort. Sci. 40: 177–186.
4. Everett, J. D. 1913. Elementary Treatise on Natural Philosophy. Based on the "Traité de Physique" of A. Privat Deschanel. 17th ed. Pp. 235–237. Blackie & Son, Ltd., London.
5. Griffiths, Dorothy G., and Nina A. Potter. 1950. Effects of ethylene upon respiratory activity of apples in gas storage, with special reference to stage of maturity. Jour. Hort. Sci. 26: 1–7.
6. Huelin, F. E. 1932. The effect of ethylene on the respiration and carbohydrate metabolism of plant tissues. Doctoral Thesis, Cambridge University, Cambridge, England.
7. Huelin, F. E., and J. Barker. 1939. The effect of ethylene on the respiration and carbohydrate metabolism of potatoes. New Phytol. 38: 85–104.
8. Michener, H. D. 1938. The action of ethylene on plant growth. Amer. Jour. Bot. 25: 711–720.
9. Platenius, H. 1942. Effect of temperature on the respiration rate and the respiratory quotient of some vegetables. Plant Physiol. 17: 179–197.
10. Pratt, H. K. 1954. Direct chemical proof of ethylene production by detached leaves. Plant Physiol. 29: 16–18.
11. Rouse, H., and S. Ince. 1957. History of Hydraulics. State Univ. of Iowa. P. 65.
12. Schwertz, F. A. 1950. Rate-indicating Mariotte bottle. Anal. Chem. 22: 1214–1216.

COMPARISON OF LIGNIN-LIKE POLYMERS PRODUCED PEROXIDATIVELY BY CINNAMIC ACID DERIVATIVES IN LEAF SECTIONS OF PHLEUM [1, 2]

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Using the quantitative techniques for lignin determination described previously for herbaceous grasses (14), the study of the production of lignin-like polymers by peroxidation of phenolic compounds such as eugenol and ferulic acid has been extended to include *p*-hydroxycinnamic and sinapic acids and coniferyl alcohol. All of these substrates give slightly different products, some of them more similar than others to the lignin isolated from mature timothy grass, *Phleum pratense* L. This lignin has been characterized by its solubility in 0.5 *N* NaOH, by an ionization difference spectrum with relatively high peaks in the 350 mμ region and smaller ones at 250 mμ and 300 mμ, a negative minimum in the 280 mμ region, and by the presence of hydroxybenzylalcohol groups (14).

These results support the general scheme (fig 5) of immediate precursors of the *p*-hydroxyl phenyl, guaiacyl, and sinapyl moieties of lignins in plants as elucidated by several groups of workers (3, 6, 10), but illustrate possible basic differences between the formation of the more highly polymerized lignin of woody plants and the smaller more soluble polymers typical of herbaceous plants.

MATERIALS AND METHODS

Substrates and material not used in the preceding paper (14) are described below. The *p*-hydroxycinnamic acid (K and K Labs.) had an m.p. of 212 to 214° C, and presumably this and all the other cinnamic acid derivatives are in the trans form. The sinapic acid was from two sources; one was kindly supplied by Dr. S. Masri of the Western Regional Research Labs. at Albany, Cal. (m.p. 186–192° C); the other was purchased from the K and K Labs. (m.p. of 186–189° C). Coniferin, the source of coniferyl alcohol, was either a sample kindly supplied by Dr. O. Goldschmid of Rayonier, Inc. (m.p. 185–188° C) or a sample from A. H. Kahlbaum, Berlin (m.p. 186–188° C). The enzyme β-glucosidase was purchased from Worthington Co., cinnamic acid (m.p 132–133° C, from Paragon Labs. and L-tyrosine from Nutritional Biochemicals Corp.

The ionization difference spectra (1) of these phenolic compounds are shown in figure 1. None of these substrates gave the typical blue color similar to that of the standard guaiacol in the 2,6-dichloroquinonechlorimide test; coniferin, coniferyl alcohol, and *p*-hydroxycinnamic acid gave negative reactions; ferulic acid produced an unstable red color, and sinapic acid formed a red color which turned to purple. Gierer has reported that the C_3 chain para to the free phenolic hydroxyl group must be split off to produce the blue azo dye, and that *p*-hydroxybenzylalcohol groups are responsible for the blue color produced by lignin preparations (7).

Coniferyl alcohol was prepared from coniferin by β-glucosidase activity and was used either directly with the enzyme still present or after ether extraction. About 36 mg of coniferin and 36 mg of β-glucosidase were incubated in 10 ml of 0.05 M KH_2PO_4 solution at pH 4.5 for 24 hours at room temperature under anaerobic conditions. This mixture was extracted with 60 ml of ether in 10 ml portions. The ether was evaporated; the residue, taken up in ethyl alcohol just before use, was analyzed quantitatively, using the data of Aulin-Erdtman (1). About 80 to 90 % of the expected coniferyl alcohol was recovered.

Green laminae (300–400 mg fresh wt) of *Phleum pratense* var. climax were cut into 0.3 to 0.5 cm long sections and floated for 24 hours on the surface of a solution containing 0.05 M KH_2PO_4 at pH 4.5 with 0.01 M phenolic substrate and 0.01 or 0.05 M H_2O_2 in a total volume of 10 ml. No detectable difference was observed between experiments using the different concentrations of H_2O_2. Controls were made with added substrate and buffer, and with buffer only. A few experiments were performed at pH 7 with phosphate buffer.

Details of the extraction techniques and the quantitative analyses are described in the preceding paper (14). After exhaustive extraction with ether and then with water, the residues were extracted overnight with 0.5 *N* NaOH at 70° C to solubilize the lignin (2). Ionization difference spectra of the alkaline extracts (1, 8) were made by subtracting the ultraviolet absorption at neutral pH (0.05 M phosphate buffer) from that at an alkaline pH (0.05 *N* NaOH); the peaks at 250 and 300 mμ indicate the presence of simple phenolic compounds, while peaks at longer wave lengths are dependent upon the presence of double bonds of the 3-carbon sidechain in conjugation with the benzene nucleus. Estimates of the free phenolic content (*p*-hydroxybenzylalcohol groups) (7) were made by means of the colorimetric 2,6-dichlorquinonechlorimide (N, 2,6-trichloro-*p*-benzequineimine) test, and the phenol values were converted to lignin values by multiplying the mg of phenol by the factor 32, derived from data on native lignin

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[2] This research has been supported by the National Science Foundation (Grant G-4651).

preparations of bagasse (14). Analyses were always made within 1 to 3 hours after the extraction was completed since some of the products were unstable after about 24 hours under alkaline conditions.

Experimental Results

Incubation With *p*-Hydroxycinnamic Acid: When *p*-hydroxycinnamic acid and H_2O_2 were added to sections of laminae of Phleum at pH 4.5, there was very little macroscopic evidence of any reaction, except for a pale brown color along the edges of the sections in the mesophyll and vascular tissue, and a slight cloudiness in the medium. When the fresh sections were viewed microscopically, brown globules could be seen in the mesophyll cells. There was only a questionable increase in the phloroglucinol histochemical test in the vascular tissue and in a pale orange-brown color in the mesophyll in the Cl_2-Na_2SO_3 test. A striking characteristic of the polymer produced from this substrate was the absence of any increase in free phenolic groups reacting in the quinonechlorimide test, a lack which was reflected in the great change in the uv/phenol ratio (table I). The difference spectrum was characterized by a negative minimum in the 280 mμ region and a high maximum at 345 mμ (fig 2), in addition to the 250 and 300 mμ peaks.

Incubation With Ferulic Acid: This reaction has been described previously (14); further data are supplied here to indicate the variability (table I and fig 2). Besides the appearance of a red color in the veins during the incubation period, pink and brown globules could be seen microscopically in many of the cells. These globules did not stain in the phloroglucinol test, and there was sometimes a slight increase in the intensity of the red color of the Cl_2-Na_2SO_3 test in the walls of the vascular tissue. Since the red color in this test is unstable and turns brown, it is

Table I

Effect of Phenolic Substrates on Alkali-Soluble Lignin Content of Phleum

Substrate	Lignin % original dry wt	uv*/phenol	ΔD/mg @ 300 mμ	ΔD/mg @ 350 mμ	Residue % original dry wt
Buffer controls					
Avg 10	11	0.43	0.21	0.38	17
Range	(8–13)	(0.3–0.5)	(0.18–0.23)	(0.27–0.45)	(12–19)
p-OH-cinnamic	11	0.39	...**	0.22	26
"	10	0.43	...	0.29	20
" + H_2O_2	10	1.4	...	1.2	31
" "	12	1.2	...	1.5	26
" "	8	1.8	...	1.2	22
Ferulic + H_2O_2	43	0.40	...**	2.1	24
" "	30	0.41	...	1.4	22
" "	29	0.43	...	1.4	22
" "	14	0.68	...	1.1	23
" "	18	0.61	...	1.8	18
Sinapic	11	0.38	0.29	0.35	19
" + H_2O_2	11	0.48	0.20	0.41	11
" "	17	0.41	0.21	0.81	19
" "	15	0.43	0.22	0.78	19
Coniferyl alcohol	14	0.38	0.31	0.43	13
" "	20	0.38	0.36	0.78	20
" " + H_2O_2	17	0.37	0.47	0.41	...
" " "	46	0.41	1.4	1.1	30
" " "	21	0.30	0.39	0.56	23
" " "	21	0.38	0.50	0.62	19
Coniferin + glucosidase + H_2O_2 ***	13	0.55	0.45	0.45	16
	12	0.49	0.40	0.44	...

* Total O.D. @ 290 mμ in 0.05 *N* NaOH/total μg of phenol calculated as guaiacol.

** Not estimated for these cinnamic acid derivatives because of the interference of the negative minimum at 280 mμ.

*** Added after preincubation with glucosidase.

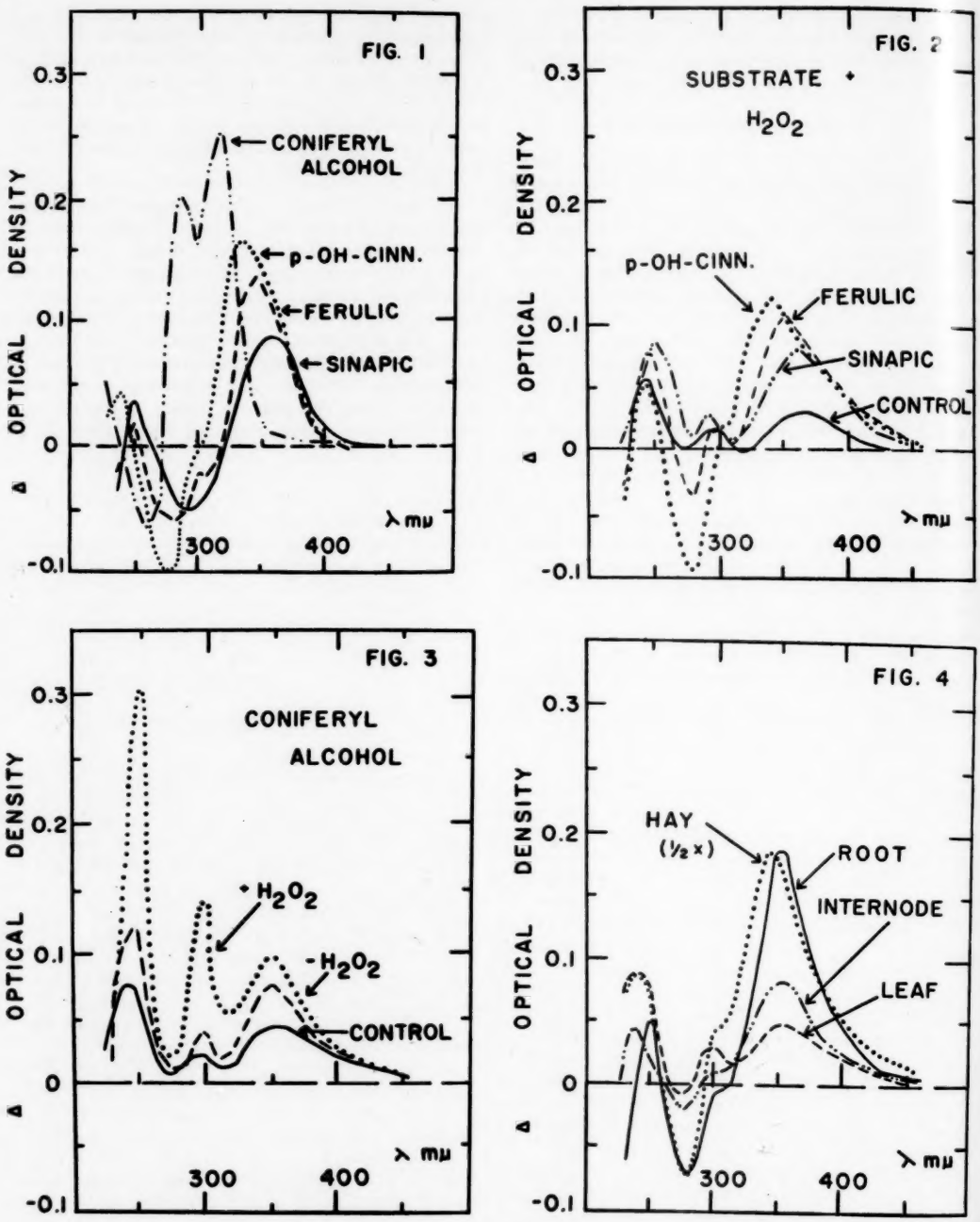

FIG. 1. Difference spectra of phenolic substrates. The Beckman cuvette contained 3.7 μg/ml of coniferyl alcohol (dashed-dotted line) or 1.5 μg/ml of *p*-hydroxycinnamic (dotted line), ferulic (dashed line), or sinapic acid (solid line).

FIG. 2. Difference spectra of extracts of leaf tissues incubated with 0.01 M *p*-hydroxycinnamic, ferulic, or sinapic acid in the presence of 0.01 M H_2O_2 at pH 4.5. Each ml of the solution in the cuvette contained an aliquot of a lignin extract equivalent to 100 μg dry weight of the original tissue.

very difficult to distinguish between the red of the syringyl-positive test and the orange-brown of the guaiacyl-positive test which likewise fades to a brown color. The major peak in the difference spectrum is generally at about 350 to 355 mμ, in between those of tissues incubated with *p*-hydroxycinnamic and sinapic acids (fig 2).

While the incubating medium with added ferulic acid at pH 4.5 remained clear and colorless during the 24 hour incubation period, the same medium at pH 7 slowly turned to a bright yellow-brown color. The difference spectrum of the two media showed no striking variations, both being similar to that of the initial substrate alone except for a slight increase in the region between 380 to 450 mμ. In contrast to the coniferyl alcohol experiment at the higher pH value, the yield of alkali-soluble product was significantly less (about half) at the higher pH.

INCUBATION WITH SINAPIC ACID: The macroscopically visible red color noted in the veins of the ferulic acid-fed-leaves was even more apparent in the case of the sinapic acid-fed-leaves. This color was also evident during the later stages of incubation with sinapic acid in the absence of added H_2O_2, but only in the internal part of the sections and never along the edges. Pink and brown globules could be observed microscopically. The incubation medium became slightly yellow and the sections showed considerable chlorophyll demage in contrast to the control sections. There was no significant increase in the phloroglucinol test, but the Cl_2-Na_2SO_3 test yielded a strong red color in the veins and an orange color in the mesophyll cells. The first wash with water removed a large proportion of the brown color of the dried sections, the orange-brown solution turning brown upon standing at room temperature. The spectrum of this aqueous solution is discussed below. The water and ether washed residue was a rich chocolate brown; this color was removed by alkaline treatment. The difference spectrum of this alkaline extract was characterized by peaks at about 250, 300, and 355 mμ and a complete absence of any negative minimum in the region of 280 mμ (fig 2).

INCUBATION WITH CONIFERYL ALCOHOL: When coniferyl alcohol and H_2O_2 were incubated with sections of laminae, there was little visual evidence of a reaction, but there was a large increase in materials reacting with phloroglucinol both in the vascular tissue as well as in the mesophyll. In the early part of the reaction, globules which stained a bright red with phloroglucinol were observed in the mesophyll cells (5). During the incubation a definite cloudiness occurred in the medium, although this was probably less than in the case of the eugenol-treated sections (14).

The feeding of coniferin for 24 hours followed by another 24 hour incubation period with added H_2O_2 produced only a small increase in the phloroglucinol-positive materials, while the addition of an external source of glucosidase greatly enhanced this reaction. These results would imply that the tissue was weak in glucosidase activity, or that the coniferin does not penetrate the cells.

In table I, data are shown for extracts of tissues pre-incubated with coniferin and β-glucosidase and then with H_2O_2, and for extracts from experiments in which coniferyl alcohol, derived from the coniferin, was added directly to the tissues in the presence of H_2O_2. The relatively high values even in the absence of added substrate and H_2O_2 should be noted; the highest set of values should be compared with the highest ones obtained in the presence of added substrate and H_2O_2 since the tissues used for incubation were selected from the same plants. As indicated by the percentage of lignin values, the product showed an increase in the free phenolic groups comparable to that obtained with ferulic acid. This alkali-soluble product from coniferyl alcohol and H_2O_2, however, differed from that obtained with ferulic acid in that there was a greater increase in the 250 and 300 mμ region of the difference spectrum than at 350 mμ (fig 3). Furthermore, a significant amount of phloroglucinol-positive polymer was insoluble in the alkaline extraction technique, a result similar to that found with eugenol (14).

When the incubation was carried out at pH 7 instead of pH 4.5 there was no significant qualitative or quantitative difference in the analysis of the alkali-soluble product. There were fundamental differences, however, in the incubation medium, with the production of more precipitate and an intense yellow color at the higher pH. The difference spectrum of this precipitate was characterized by large peaks at 250 and 300 mμ and a definite shoulder at about 320 mμ. The spectrum of the supernatant showed a major peak at 300 mμ, secondary ones at 250 and 320 mμ, and a low but definite peak at about 400 mμ. This indicates a definite change from the original spectrum of coniferyl alcohol with peaks at about 230, 290, and 320 mμ (fig 1). The medium at pH 4.5 showed a difference spectrum approximately intermediate between that of coniferyl alcohol and the pH 7 medium. Siegel (11)

FIG. 3. Difference spectra of extracts of leaf tissue incubated with 0.01 M coniferyl alcohol with (dotted line) and without (dashed line) H_2O_2 and the control with buffer alone (solid line). Each ml of solution in the cuvette contained an aliquot of lignin extract equivalent to 100 μg dry weight of the original tissue.

FIG. 4. Difference spectra of extracts of different organs of Phleum. Each ml of solution in the cuvette contained an aliquot of lignin extract equivalent to 100 μg of the original dry weight of the hay, or 200 μg of the original dry weight of young roots, first internodes, and leaves. The lignin content, expressed in percent of the original dry weight and based on the quinonechlorimide test, was 16 % for hay, 14 % for the roots, 4 % for the first internode, and 6 % for the green leaves.

reported a smaller yield and an intensification of a yellow coloration in the medium with eugenol at pH values higher than 4.5. Freudenberg (4) indicated that there are major differences in the type of dimeric precursor and the subsequent polymer produced at pH 7 and pH 5.5; he generally prepared his synthetic lignin or "DHP" at the higher pH value.

INACTIVE SUBSTRATES: When L-tyrosine and cinnamic acid were used as the substrates in these incubation experiments, no detectable lignin products were obtained. Since the radioactive tracer work of Brown and Neish (3) demonstrated that these are precursors of lignins in grasses, these negative results may indicate that the conversion of these compounds to *p*-hydroxycinnamic acid is not rapid enough, that the enzymatic reactions involved are inhibited by the high concentration of H_2O_2, or that the substrates are being metabolized to a non-lignin product.

WATER SOLUBLE PRODUCTS: When sinapic acid was incubated with laminae, a large proportion of the product was soluble in water; this result led to further examination of these extracts (table II). The difference spectrum showed a large peak in the 260 mμ region with a slightly smaller one at 380 mμ. Extracts from control experiments showed small, irregular peaks at 250 mμ and 300 mμ with a slightly higher peak in the 380 mμ region. When extracts of ferulic acid-fed-leaves were examined, some of these showed a definite increase in the 380 mμ region; this increase in a water-soluble component was generally associated with lower values for the 350 mμ peak in the alkaline extracts. Extracts of coniferyl alcohol-fed-leaves showed slight increases in the 250 and 300 mμ peaks, but no significant change in the 380 mμ region. The ether extracted materials have not been analyzed.

THE 350 MΜ PEAK: Since the high peak at approximately 350 mμ in the difference spectrum is characteristic of the lignin of mature timothy, the difference spectra of extracts from various organs of the growing grass plant were examined.

While young and old laminae had a less pronounced peak at 350 mμ, the internodes and especially the roots of young Phleum seedlings (2–3 weeks old) exhibited the pronounced absorption at 350 mμ found in mature shoots or hay (fig 4). In all cases, the high peak at 350 mμ was associated with a pronounced minimum at 280 mμ, an increase in the phenol-reacting groups in the quinonechlorimide test, and tissue having the strongest phloroglucinol and Cl_2-Na_2SO_3 tests.

A definite but variable amount of the product producing the absorption in the difference spectra at regions greater than 320 mμ could be precipitated with acid as described in the method of Bondi and Meyer (2). The difference spectrum of the precipitate had peaks which were broader and were shifted toward the longer wave lengths up to 400 mμ, and the phenolic content was lower and the uv/phenol ratio was higher than that of the original extract. The product of the reaction with coniferyl alcohol and H_2O_2 was the most easily precipitated with acid.

When a hay sample was exhaustively extracted with ethyl alcohol prior to the alkaline extraction, there was no significant difference in the subsequent analyses of this alcohol-extracted residue from the non-alcohol extracted material. The alcohol extracts, on the other hand, showed no indication of any lignin since the major peak of the difference spectrum was at 410 mμ with a smaller one at 270 mμ. The spectrum remained unchanged even if this alcohol extract was heated at 70° C under alkaline conditions.

DISCUSSION

The characteristics of the lignin-like polymers produced by peroxidation of five closely related phenolic compounds (fig 5) are summarized in table III, and are compared with those of the lignin isolated from timothy hay. On the basis of these characteristics, ferulic acid produces the most natural lignin, but the product reacts weakly in the histochemical phloroglucinol test. Coniferyl alcohol, on the other hand, is very effective in increasing the histochemical phloroglucinol reaction in both mesophyll and vascular tissue, but a major portion of this product is not soluble in 0.5 *N* NaOH, and therefore, may be considered an unnatural form of lignin in timothy. Similar results were obtained previously with eugenol (14).

The discrepancy between the results obtained with ferulic acid and coniferyl alcohol is of considerable interest, because the former substrate is converted to its alcohol (i.e., coniferyl alcohol) before it is oxi-

TABLE II

WATER SOLUBLE PRODUCTS OF PEROXIDATIC ACTION ON PHENOLIC SUBSTRATES

	H_2O SOLUBLE	ALKALI SOLUBLE
	ΔD/MG @ 380–390 MΜ	ΔD/MG @ 350 MΜ
Buffer	0.23	0.34
p-Hydroxycinnamic acid + H_2O_2	0.19	1.2
Ferulic acid + H_2O_2	0.38	1.1
" " "	0.14	1.8
Sinapic acid + H_2O_2	0.42	0.78
" " "	0.54	0.81

TABLE III

COMPARISON OF MAJOR CHARACTERISTICS OF LIGNIN-LIKE POLYMERS PRODUCED PEROXIDATIVELY AT pH 4.5 BY GREEN LEAVES AND THE LIGNIN OF HAY

	Substrates fed to green leaves					Hay
	p-OH-Cinnamic	Ferulic	Sinapic	Coniferyl alcohol	Eugenol	
Color reaction in veins						
Visible red	...	++	+++	...	...	...
Phloroglucinol test	?	+	?	++++	++++	++*
Cl_2-Na_2SO_3 test**	...	?	+++	...	...	++
H_2O Extract						
Δ D @ 380 mμ	...	++	++++	...	...	( )***
Alkaline extract						
Δ D @ 250 mμ	++	++	++	++++	+++	++
Δ D @ 300 mμ	++	++	++	++++	+++	++
Δ D @ 350 mμ	++++	++++	+++	++	+	++++
Δ D @ 270 mμ†	+++	+++	...	...	...	++++
Quinonechlorimide test	...	++++	++	++++	++	++++
Alkali insoluble residue						
Phloroglucinol test	...	...	...	+++?	++++	...

* As compared with gymnosperm wood (++++)
** Red color
*** H_2O extract very dark brown; not analyzed
† Negative minimum

datively polymerized according to the scheme elucidated by Brown and Neish (3) and Freudenberg (6) (fig 5). The major differences in the products of these two substrates are in the amount of the phloroglucinol-reacting substances, the amount of material absorbing in the region above 310 to 320 mμ in the difference spectrum, and the solubility of the polymer produced. These variations may reflect a basic difference between the lignins of gymnosperm wood and the herbaceous grasses, for although coniferyl alcohol yields some measurable amount of alkali-soluble product in Phleum, its difference spectrum is closer to that found in the enzymatically produced dehydrierungspolymerizat (DHP) and to that of lignin extracts of gymnosperm woods (5). Likewise, the solubility characteristics of the phloroglucinol-reacting material are closer to those expected of woody gymnosperm tissues.

Several interpretations of these experimental results can be considered. First, some of the ferulic acid may be converted to sinapic acid before polymerization via sinapyl alcohol, producing a more soluble polymer, such as the one described by Freudenberg (5, 6). Second, ferulic acid may be converted into lignanes (4) or directly into a more soluble ferulic acid lignin in the case of the Phleum tissue. Freudenberg reports that such a direct conversion is not the case at least for some of the lignin produced in gymnosperm tissue (4). Third, the polymer formed may differ with the concentration of free coniferyl alcohol at any one time, the concentration being initially higher in the case of the added coniferyl alcohol than with added ferulic acid. Again, Freudenberg reports differences in the characteristics of DHP depending on whether the substrate is added all at once or drop by drop (4).

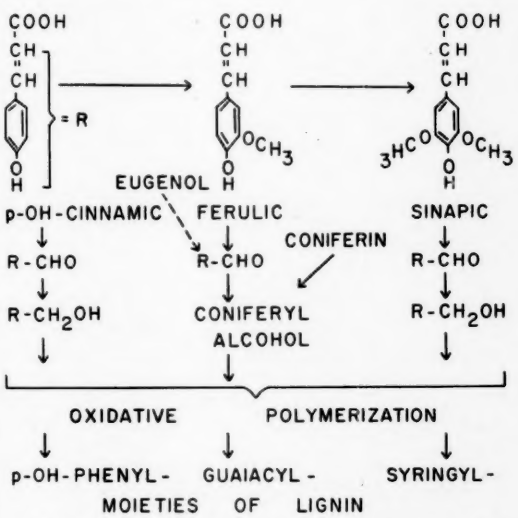

FIG. 5. Summary of the postulated pathway of lignin biosynthesis as elucidated by several groups of workers (3, 6, 10).

Another basic problem is the significance of the difference spectrum in the region of 350 mμ. Such high peaks at wave lengths greater than 310 mμ are not typical of woody plants, although small peaks are sometimes evident (8, 14). Similar peaks, however, have been found in other grasses, i.e., in native lignin preparations from bagasse and wheat (13, 14). Absorption above 310 mμ could be due to the presence of double bonds in the side chains in conjugation with free phenolic nuclei, indicating less highly polymerized molecules or ester linkages (12). Preliminary work on lignins precipitated by acid indicates that the materials absorbing in this portion of the spectrum are complex. If any of this absorption at 350 mμ is due to a contaminating non-cell wall material, this contaminant is insoluble in alcohol, ether, and water. The degree of alteration of the original lignin polymer during the alkaline extraction is not known.

Besides the problem of the non-physiological conditions necessary to produce these lignin-like polymers (14), and the lignification in some cases of non-vascular tissue, further difficulties in the interpretation of these results may be due to confusion concerning the nature of lignin, our knowledge of which is based largely on data from the highly polymerized lignins of woody tissues. Beyond the characterization of lignin as a constituent of cell walls composed of polymers of phenylpropane units with some phloroglucinol-reacting groups, there may be taxonomic differences besides the variation in the amount of the three basic phenylpropane units (9). Furthermore, the various histochemical stains used to identify lignin, such as safranin, phloroglucinol, and Cl_2-Na_2SO_3, do not always agree when tracheids and sclerenchyma, and when tissues produced by primary and by secondary growth, are compared (15). More work is necessary with lignins from sources other than woods to bridge the gap between our morphological and chemical information concerning this aromatic polymer.

Summary

A series of cinnamic acid derivatives and related compounds postulated as lignin precursors were incubated with sections of the lamina of *Phleum pratense* (timothy grass) in the presence of H_2O_2 at pH 4.5. Cinnamic acid and tyrosine were inactive, while *p*-hydroxycinnamic, ferulic, and sinapic acids, and coniferyl alcohol produced lignin-like polymers, presumably by peroxidative polymerization. The actual products, however, differed in their reaction to histochemical tests for lignin, in their solubility in water and in 0.5 *N* NaOH, in the free phenolic groups detected by the quinonechlorimide test, and in their ionization difference spectra. When compared with the lignin extracted from mature timothy shoots or hay, ferulic acid produced the most natural product. Although these results support the general scheme implicating these compounds as immediate precursors of the three different moieties found in grass lignins, the variations in the polymers formed may reflect basic differences in the solubility and polymerization of lignins from woody plants and from herbaceous grasses.

Literature Cited

1. Aulin-Erdtman, G., and L. Hegbom. 1957. Spectrographic contributions to lignin chemistry. VII. The ultraviolet absorption and ionization Δε-curves of some phenols. Svensk Papperstidn. 60: 671–681.
2. Bondi, A., and H. Meyer. 1948. Lignins in young plants. Biochem. Jour. 43: 248–256.
3. Brown, S. A., and D. Wright, and A. C. Neish. 1959. Studies of lignin biosynthesis using isotopic carbon. VII. The role of *p*-hydroxyphenlpyruvic acid. Can. Jour. Biochem. Biophys. 37: 25–34.
4. Freudenberg, K. 1956. Beiträge zur Erforschung des Lignins. Angew. Chem. 68: 508–512.
5. Freudenberg, K. 1959. Biochemische Vorgänge bei der Holzbildung. In: Biochemistry of Wood. Vol. II. Pp. 121–136. 4th Intern. Cong. Biochem., Vienna. Pergamon Press, New York.
6. Freudenberg, K. 1959. Biosynthesis and constitution of lignin. Nature 183: 1152–1155.
7. Gieper, J. 1954. Die reaktion von Chinonmonochlorimid mit Lignin. I. Spezifität der Reaktion auf *p*-oxybenzylalcoholgruppen und deren Bestimmung in verschiedenen Lignin präparaten. Acta Chemica Scand. 8: 1319–1331.
8. Goldschmid, O. 1954. Determination of phenolic hydroxyl content of lignin preparations by ultraviolet spectrophotometry. Anal. Chem. 26: 1421–1423.
9. Manskaja, S. M. 1959. Zur phylogenese des Lignins. In: Biochemistry of Wood. Vol. II. Pp. 215–226. 4th Intern. Cong. Biochem., Vienna. Pergamon Press, New York.
10. Schubert, W. J., S. N. Acerbo, and F. F. Nord. 1957. Investigations on lignin and lignification. XVIII. Incorporation of *p*-hydroxyphenylpyruvic acid into lignin. Jour. Amer. Chem. Soc. 79: 251–252.
11. Siegel, S. M. 1954. Studies on the biosynthesis of lignin. Physiol. Plantarum 7: 41–50.
12. Smith, D. D. C. 1955. Ester groups in lignin. Nature 176: 267–268.
13. Smith, D. D. C. 1955. Contribution of residues containing carbonyl to the ultra-violet absorption of lignins. Nature 176: 927–928.
14. Stafford, H. A. 1960. Differences between the lignin-like polymers formed by peroxidation of eugenol and ferulic acid in leaf sections of Phleum. Plant Physiol. 35: 108–114.
15. Wardrop, A. B. 1957. The phase of lignification in the differentiation of wood fibers. Tappi 40: 225–243.

CHLOROSIS IN SOYBEAN AS RELATED TO IRON, PHOSPHORUS, BICARBONATE, AND CYTOCHROME OXIDASE ACTIVITY [1]

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Many investigators have reported reduced iron accumulation in the shoots of plants when grown in calcareous soil or bicarbonate solutions (3, 5, 11, 19). Correlated with decreased iron content in shoots, Thorne and associates (7, 18, 20) have found iron accumulation in the roots. These results suggest iron immobilization within the chlorotic plant and reduced translocation to the leaves.

It has been established that bicarbonate ions are involved in lime-induced chlorosis. Lindsay and Thorne (11) reported higher concentrations of bicarbonate and calcium plus magnesium from soil solutions of chlorotic areas than adjacent non-chlorotic areas. Porter and Thorne (16) conducted nutrient culture experiments in which pH and bicarbonate ion concentrations were varied by regulating the CO_2 pressure of the aeration stream. Chlorosis was induced at the higher bicarbonate concentrations, but high pH alone did not produce chlorosis. Goss (8) compared the influence of bicarbonate with chloride, nitrate, and sulfate on the uptake and translocation of certain mineral elements. Bicarbonate significantly decreased the uptake and translocation of mineral elements in comparison to the other anions used. Miller and Thorne (15) found the respiration of excised roots from chlorosis-susceptible plants to be inhibited by bicarbonate, whereas the respiration of roots from chlorosis-resistant plants was only slightly affected. Subsequent results by Miller and Evans (13) indicated that cytochrome oxidase was inhibited by bicarbonate. Reduced cytochrome oxidase activity was observed in extracts from roots of bicarbonate-induced chlorotic soybeans when compared to control green plants (14).

The afore-mentioned results support the hypothesis that bicarbonate is absorbed by the plant and affects the metabolism, conceivably by acting upon iron enzymes such as cytochrome oxidase. Other experiments support the contention that the effect of bicarbonate is external on the growing medium and not direct. In a split-medium experiment in which the roots of chlorosis-susceptible PI-54619-5-1 soybeans (PI) were allowed to grow through a soil mix and air gap into a solution culture, adding large amounts of bicarbonate to the solution culture did not induce chlorosis (1). Iron was absorbed from the soil and bicarbonate was in the solution culture. Under these experimental conditions, bicarbonate had no apparent internal effect on plant metabolism. Bicarbonate increases the solubility of phosphorus in solution (9), and Brown et al have demonstrated an internal inactivation of iron in plants by phosphorus, resulting in chlorosis (2). They (2) concluded that the effect of bicarbonate on iron chlorosis is an indirect effect of this ion on phosphorus and calcium availability, and chlorosis is related more to phosphorus concentration than bicarbonate concentration of the nutrient solution.

In order to elucidate the role of bicarbonate in chlorosis, plants that were subjected to various experimental nutrient conditions were analyzed for cytochrome oxidase activity, iron and phosphorus content in the tissue.

MATERIALS AND METHODS

EXPERIMENT I. A split-medium experiment was designed to determine the influence of bicarbonate and phosphorus on cytochrome oxidase activity and chlorosis when iron was not in direct contact with bicarbonate or phosphorus. The design permitted roots of Hawkeye (HA) and PI-54619-5-1-(PI) soybeans to extend from a soil mixture through an air gap into a solution culture containing 10 meq/l of $NaHCO_3$ or NaCl (1). The soil mixture contained by glass wool and plastic screen in an inverted 300 ml Erlenmeyer flask with the bottom removed, was composed of a mixture of calcareous and organic soil known to supply approximately the minimum quantity of iron to prevent chlorosis in the soybean. When the plant roots had grown through the soil and protruded below the plastic screen, the Erlenmeyer flasks containing the growing plants were placed in the holes of a wooden lid over a 9 liter pyrex battery jar containing nutrient solution. The effect of phosphate added to the nutrient solution in the presence and absence of bicarbonate was correlated with chlorosis.

The solutions contained the following concentrations of minerals in meq/l, Ca 1.8, Mg 0.4, NO_3 2.5, and K 0.1. The treatment variables consisted of additions to the above nutrient solutions as noted in table I. The pH values of the bicarbonate treatments were maintained at pH 7.8 by aerating with 1 percent CO_2 in air. Sodium hydroxide was added several

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TABLE I

TREATMENT VARIABLES FOR EXPERIMENT 1 (SPLIT MEDIUM, SOIL-NUTRIENT SOLUTION)

TREATMENT NO.	MEQ/L $NaHCO_3$	MEQ/L NaCl	PPM P	AERATION
1	0	10	0.05	Air
2	0	10	0.15	Air
3	0	10	0.60	Air
4	0	10	2.40	Air
5	10	0	0.05	Air + 1.0 % CO_2
6	10	0	0.15	Air + 1.0 % CO_2
7	10	0	0.60	Air + 1.0 % CO_2
8	10	0	2.40	Air + 1.0 % CO_2

times daily to the NaCl treatments to adjust the pH to 7.8 (At pH 7.8 the NaCl treatments would contain less than 1 meq/l of $NaHCO_3$). Well over 50 percent of the root growth in each case was in the solution part of the medium. This portion of the roots was used for cytochrome oxidase assay. Plants were harvested 7 to 9 days after treatment initiation.

EXPERIMENT II. A split-root experiment was designed to determine the same factors as in experiment I, but under conditions in which HA and PI soybeans were grown entirely in solution culture. The soybeans were germinated on a steel tray covered with water-saturated cheese cloth. After several days the primary root tip was removed, leaving eight lateral roots. The plant roots were suspended in nutrient solution containing the following elements expressed in meq/l: 1.3 Ca, 0.3 Mg, 2.5 N, 1.0 K, 1.0 P, and 0.13 SO_4. The plants grew in this solution until the roots were 2 to 3 inches long, and were then transferred to treatment solutions. Each plant was suspended over two containers with the roots equally divided between the pair of containers. All containers had certain common nutrients, but phosphorus and bicarbonate were added to one container of each pair and iron and other minor elements were added to the other container. This arrangement enabled the plants to absorb all required nutrients and eliminated direct contact in the solution between iron and bicarbonate and phosphorus. Each container had the following common nutrients in meq/l: Ca 1.3, Mg 1.3, NO_3 2.0, K 0.5, and S 0.13. Minor elements were added to one of the containers of each pair in the following concentrations in ppm: Fe 1.0 (present as $FeCl_3$), Mn 0.7, B 0.04, Zn 0.02, Cu 0.005, and Mo. 0.005. Phosphorus was added to the other container, which contained no minor elements at four levels, 0.05, 1.10, 3.35, and 6.67 ppm. Sodium bicarbonate (10 meq/l) was added to all the pots containing phosphorus and these containers were maintained at pH 7.8 by aeration with a mixture of 1 percent CO_2 in air. The solutions containing the minor elements were adjusted daily to pH 6.0 using NaOH. Plants were harvested 11 to 12 days after treatment initiation. Extracts for assaying cytochrome oxidase activity were prepared from roots of the soybean varieties growing in the phosphorus-bicarbonate nutrient solution.

EXPERIMENT III. The split-root technique using nutrient solution as described in the previous experiment was utilized to determine the influence of phosphorus independent of bicarbonate on chlorosis, cytochrome oxidase, and iron in terms of phosphorus in solution and phosphorus in the tissue. Two levels of phosphorus, 0.05 and 6.7 ppm were added to the containers having no minor elements. Ten meq/l of $NaHCO_3$ or NaCl was also added to these containers. The treatment variables are shown in table II. This experiment was designed to include NaCl in order to elucidate any direct HCO_3 effect. Solutions which contained 10 meq/l $NaHCO_3$, were maintained at pH 7.8 by aerating with a mixture of 1 percent CO_2 in air. Solutions which contained 10 meq/l NaCl, were maintained at pH 7.8 by daily additions of NaOH. Nutrient solutions containing minor elements were adjusted to pH 6.0 with daily additions of NaOH. Plants were harvested 11 to 12 days after treatment initiation. Extracts for cytochrome oxidase assays were prepared from roots growing in solutions containing phosphorus and NaCl or $NaHCO_3$.

TABLE II

TREATMENT VARIABLES FOR EXPERIMENT 3 (SPLIT-ROOT, NUTRIENT CULTURE)

TREATMENT NO.	MEQ/L $NaHCO_3$	MEQ/L NaCl	PPM P	AERATION
1	10	0	0.05	Air + 1.0 % CO_2
2	10	0	6.7	Air + 1.0 % CO_2
3	0	10	0.05	Air
4	0	10	6.7	Air

EXPERIMENT IV. To compare the influence of bicarbonate and phosphorus on chlorosis, cytochrome oxidase activity, iron content in the leaves, phosphorus content in solution and in the leaves, where iron was subjected to external influences of pH and phosphorus; soybeans were grown in conventional solution cultures contained in 9-liter pyrex battery jars. Two PI and two HA soybean plants were grown per jar in aerated solution culture supported by cotton plugs. The nutrient solution consisted of the following concentration of minerals (meq/l): Ca 2, Mg 1, K 2.2, NH_4 0.5, NO_3 5.2, SO_4 0.5; (ppm) P 0.6, Fe 7.5 (added as $FeCl_3$), Mn 0.08, B 0.04, Zn 0.03, Cu 0.01, Mo 0.01, and Cl 5.0.

The treatment variables consisted of additions to the nutrient solution as found in table III.

From previous experimental data (1), the levels of micro-nutrients and phosphorus (0.6 ppm) were selected at low values so as not to enhance the expression of iron deficiency. Enough iron was added (7.5 ppm) to supply the PI plants with sufficient iron under normal conditions to remain green. All solutions were maintained at pH 7.8. Aerations with CO_2 maintained the bicarbonate solutions at the proper pH. Additions of NaOH to the NaCl solutions were necessary several times daily, but even then the solutions reached pH values as low as pH 7.0. Plants were harvested 8 to 9 days after treatment initiation.

ANALYTICAL METHODS. Plants were harvested when the second trifoliate leaves were fully expanded. Treatments in all experiments were replicated four times and the values averaged. Maximum deviation from the mean was 10 percent and in most cases much less.

Iron was determined from plant material on a dry weight basis using the orthophenanthroline method (17). Phosphorus was determined in the culture solution by modification of Dickman and Bray (6) and in plant tissue on a dry weight basis by the vanadate procedure (10). The culture solution was filtered prior to P analysis.

CYTOCHROME OXIDASE ASSAY. Particulate preparations used in the various experiments were made from the roots of PI or HA soybean. Extracts of root tissue were prepared as outlined previously (13). One gram wet weight of roots was ground in a cold mortar with 20 ml cold Tris buffer and then homogenized with a Ten Broeck homogenizer and centrifuged at 1,000 × G for 15 minutes. The supernatant solution was decanted and centrifuged at 20,000 × G for 15 minutes. Particulate material was collected and suspended in 5 ml cold 0.05 M Tris buffer at pH 7.4. Extracts prepared by this procedure contained 0.5 mg protein per ml as estimated, using Folin's phenol reagent (12). Reduced cytochrome c (Sigma Chemical Co. of St. Louis, Mo.) was prepared chemically by the use of sodium hydrosulphite (4) and the assays were determined spectrophotometrically at 550 mμ with a Beckman DU spectrophotometer (4). The reaction mixture in a final volume of 1 ml contained 50 μ moles tris-hydroxymethylaminomethane (Tris) buffer at pH 7.4, 0.051 μ moles reduced cytochrome c, and extract containing 0.05 mg protein. Enzyme activity is indicated as the decrease in optical density at 550 mμ per mg protein between 15 and 75 seconds after the reaction was started by the addition of the enzyme.

EXPERIMENTAL RESULTS

EXPERIMENT I. The development of chlorosis in PI soybean plants, which were subjected to growth in a split-medium (soil-nutrient solution) was primarily influenced by the phosphorus concentration. When the nutrient solution contained less than 0.6 ppm phosphorus no chlorosis developed in the absence (NaCl replacement) or presence of 10 meq/l $NaHCO_3$. At higher phosphorus levels chlorosis developed in both the absence and presence of $NaHCO_3$; but in general, chlorotic symptoms appeared sooner

TABLE III

TREATMENT VARIABLES IN EXPERIMENT 4 (NUTRIENT SOLUTION)

TREATMENT NO.	MEQ/L $NaHCO_3$	MEQ/L NaCl	AERATION
1	0	0	Air
2	1	0	Air + 0.10 % CO_2
3	2	0	Air + 0.20 % CO_2
4	4	0	Air + 0.40 % CO_2
5	6	0	Air + 0.60 % CO_2
6	10	0	Air + 1.0 % CO_2
7	0	1	Air
8	0	2	Air
9	0	4	Air
10	0	6	Air
11	0	10	Air

when NaCl replaced the $NaHCO_3$. HA soybeans did not develop severe chlorosis under any of the treatment conditions used.

Less iron was found in the leaves of PI than HA soybeans at all phosphorus concentrations as shown in figure 1B. Iron concentration in the leaves showed no significant decrease as the phosphorus concentration in solution culture increased, although a trend in this direction is indicated.

More phosphorus accumulated in the leaves of plants growing in solution cultures containing NaCl than those containing $NaHCO_3$ (fig 1C). In contrast to this, higher concentrations of soluble phosphorus were found in solution cultures containing $NaHCO_3$ than those containing NaCl (fig 1D).

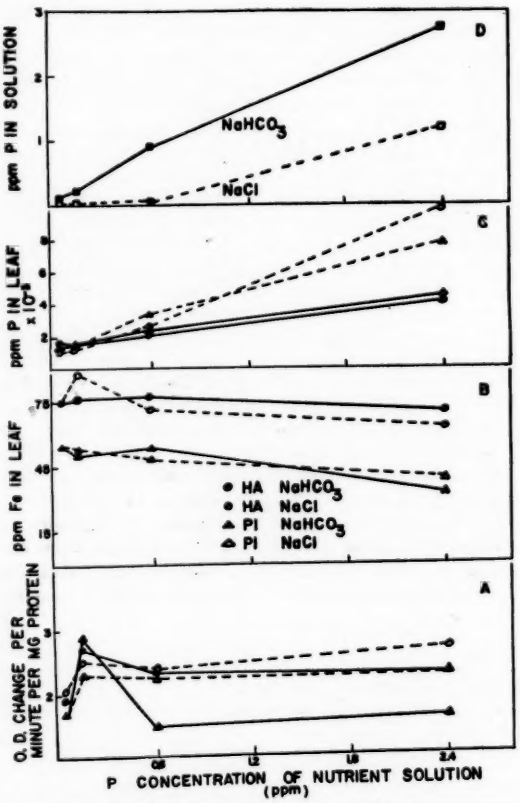

FIG. 1. Effect of P added to the solution culture containing 10 meq/l $NaHCO_3$ or NaCl on the following: A. Cytochrome oxidase activity of extracts from roots of PI and HA soybeans, L.S.D. (5%) 0.62. B. Concentration of Fe in the leaves of HA and PI soybean (dry wt basis). C. Concentration of P in the leaves of PI and HA soybeans (dry wt basis), and D. Soluble P in solution culture.

The legend for the four curves in figures A, B, and C is found in figure B.

Details of this experiment are found under experiment I in Materials and Methods.

Cytochrome oxidase activity of PI roots (10 meq/l $NaHCO_3$) was greater when roots extended from the soil mixture into solutions containing 0.15 ppm phosphorus (fig 1A) than in 0.05 or 0.6 ppm phosphorus and above; the enzyme activity of PI root extracts from bicarbonate treatments showed less activity than HA extracts or PI extracts from chloride treatments. Cytochrome oxidase activity was more related to phosphorus concentration in solution culture than to either NaCl or $NaHCO_3$ treatments (fig 1A); however, at higher phosphorus levels with PI soybean, reduced cytochrome oxidase activity was correlated with the bicarbonate treatment. No reduction in enzyme activity was observed in the comparative chloride treatment (fig 1A).

EXPERIMENT II. When soybean plants were subjected to a split-root experiment which allowed the uptake of iron without the external combined influence of phosphorus and bicarbonate, no chlorosis developed at a phosphorus level of 0.05 ppm; however, when 1.10 ppm phosphorus or more was present in the culture solution, chlorosis developed in the PI but not the HA soybeans. Increasing phosphorus levels with a constant $NaHCO_3$ level resulted in a decrease in total iron content found in PI leaves, but had little effect on the iron content of HA leaves (table IV). Both varieties of soybeans contained more phosphorus on a dry weight basis in the leaf tissue at higher phosphorus concentrations than at the lower concentrations. Cytochrome oxidase activity of extracts from soybean roots was reduced at the minimum phosphous concentration and was also reduced at the highest phosphorus level in the PI soybeans in the presence of $NaHCO_3$.

EXPERIMENT III. Soybean plants were subjected to a split-root experiment which allowed the uptake of iron without external influences of both phosphorus and bicarbonate and with phosphorus alone. Sodium chloride substituted for $NaHCO_3$ in half of the phosphorus treatments. Chlorosis developed at the higher phosphorus level in the presence of $NaHCO_3$ or NaCl. Increasing the phosphorus concentration of the nutrient solution resulted in less iron in leaves and roots of PI soybeans, and to some extent in the leaves of the HA variety (table V). No significant difference was attributable to the NaCl or the $NaHCO_3$ treatment. Phosphorus accumulated both in leaf and root tissue with increased phosphorus levels in the nutrient solution. Cytochrome oxidase activity was affected by phosphorus concentrations as shown in table V, and as observed in experiment I; at higher phosphorus concentrations with PI soybeans, reduced cytochrome oxidase activity was correlated with the bicarbonate treatment.

EXPERIMENT IV. All the results reported thus far referred to split-medium or split-root experiments (exp. 1, 2, 3) in which iron was supplied separated from the precipitating influence of pH, bicarbonate, and phosphorus. Experiment 4 was conducted for

TABLE IV

EFFECTS OF VARIOUS CONCENTRATIONS OF P ADDED TO SOLUTION CULTURES ON CYTOCHROME OXIDASE ACTIVITY AND CONCENTRATIONS OF FE AND P IN TISSUE FROM PLANTS GROWING IN SPLIT-ROOT CULTURES CONTAINING 10 MEQ/L $NaHCO_3$ *

P PRESENT IN SOLUTION (PPM)	CYTOCHROME OXIDASE ACTIVITY FROM ROOT TISSUE** O.D. CHANGE/MIN/MG PROTEIN		PPM FE IN LEAF TISSUE		PPM P IN LEAF TISSUE	
	HA	PI	HA	PI	HA	PI
0.05	2.74	2.70	54	55	1,346	522
1.10	4.06	2.90	59	17	3,341	1,876
3.35	3.79	3.38	40	22	2,970	2,145
6.70	3.95	2.70	55	10	6,578	3,244

* Details of this experiment are outlined under experiment II in Materials and Methods.
** Standard assay procedure was used as described in Materials and Methods. One-tenth ml of soybean extract containing 50 μg protein was used as the enzyme. L.S.D. (5 %) 0.65.

comparative purposes in which iron was subjected to the external precipitating influences of pH, bicarbonate, and phosphorus by including them all in the same solution culture. Under these conditions at a constant level of 0.6 ppm phosphorus, chlorosis developed in PI soybeans with as little as 2 meq/l $NaHCO_3$ in the solution culture. Iron concentration in PI and HA leaves was lower from $NaHCO_3$ than NaCl treatments. PI leaves contained less than 40 ppm iron when harvested from treatments containing at least 2 meq/l $NaHCO_3$ (fig 2B). HA soybeans were non-chlorotic in all treatments at the time of harvest and contained more than 50 ppm iron. Both PI and HA soybeans were non-chlorotic on the NaCl treatments.

PI and HA leaves from plants grown on solution cultures containing NaCl contained about the same phosphorus concentration as leaves from bicarbonate treatments (fig 2C). Although the initial addition of phosphorus to the various solution culture treatments was the same, increasing the concentration of bicarbonate increased the soluble phosphorus proportionately. At 10 meq/l there was 10 times more soluble phosphorus present in the bicarbonate than chloride solutions (fig 2D).

DISCUSSION

All the experiments involving the split-medium and split-root techniques showed rather conclusively that bicarbonate had very little direct effect upon chlorosis when iron, phosphorus, bicarbonate, and calcium were not in direct contact with each other. Chlorosis developed in plants growing under high levels of phosphorus in the presence or absence of bicarbonate. This may be explained by the increased phosphorus concentration found in these plant materials, resulting in possible internal iron inactivation. The development of chlorosis in PI soybeans under split-root or split-medium experiments was related more to phosphorus concentration in the plant material than to bicarbonate concentration of the growth medium. At equal phosphorus in $NaHCO_3$ or NaCl treatments, both PI and HA soybean leaves contained more phosphorus from NaCl than $NaHCO_3$ treat-

TABLE V

EFFECT OF P ADDED TO SOLUTION CULTURE ON CYTOCHROME OXIDASE ACTIVITY AND CONCENTRATIONS OF FE AND P IN TISSUE FROM PLANTS GROWING IN SPLIT-ROOT CULTURES CONTAINING 10 MEQ/L $NaHCO_3$ OR NACL *

SALT ADDITIVE TO SOLUTION CULTURE 10 MEQ/L	P PRESENT IN SOLUTION (PPM)	CYTOCHROME OXIDASE ACTIVITY FROM ROOT TISSUE ** O.D. CHANGE/MIN/MG PROTEIN		PPM FE IN LEAF TISSUE		PPM P IN LEAF TISSUE		PPM FE AND P IN PI ROOT TISSUE	
		HA	PI	HA	PI	HA	PI	FE	P
$NaHCO_3$	0.06	2.60	2.60	67	61	1,280	1,355	167	1,760
	6.70	3.92	2.90	51	40	6,000	6,370	59	4,850
NaCl	0.06	3.24	3.00	61	55	1,350	1,625	110	1,750
	6.70	3.52	3.64	41	45	7,000	7,450	50	8,150

* Details of this experiment are outlined under experiment III in Materials and Methods.
** Standard assay procedure was used as described in Materials and Methods. One-tenth ml of soybean extract containing 50 μg protein was used as the enzyme. L.S.D. (5 %) 0.59.

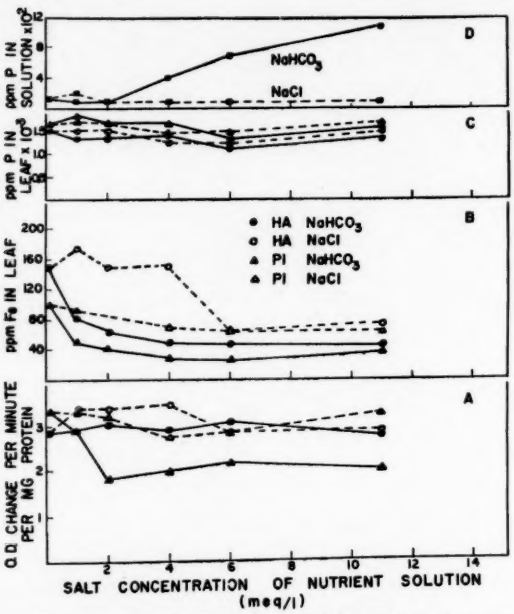

FIG. 2. The effect of NaCl and $NaHCO_3$ added to solution culture (no split-root or split-medium experiment) on the following: A. Cytochrome oxidase activity of extracts from roots of PI and HA soybeans, L.S.D. (5 %) 0.45; B. Concentration of Fe in the leaves of PI and HA soybeans (dry wt basis); C. Concentration of P in the leaves of PI and HA soybeans (dry wt basis); and D. Soluble P in the solution culture.

The legend for the four curves in figures A, B, and C is found in figure B.

The details of this experiment are found under experiment IV in Materials and Methods.

ments, even though $NaHCO_3$ cultures contained three to four times more phosphorus in solution. Bicarbonate appeared to interfere or to compete with phosphorus absorption either directly or indirectly. The indirect effect might result from a less available phosphorus compound formed by $NaHCO_3$ (1).

In experiments using complete solution cultures where iron, phosphorus, calcium, and bicarbonate were in direct contact with each other, chlorosis was directly related to the concentration of $NaHCO_3$ and the resulting high phosphorus concentration in the solution culture, which occurred with higher bicarbonate treatments. Under these conditions the higher concentration of soluble phosphorus in the $NaHCO_3$ treatments compared to the NaCl treatments may have externally precipitated iron, thus inducing chlorosis.

Cytochrome oxidase activity in extracts prepared from PI and HA roots was as great in roots grown in 10 meq/l $NaHCO_3$ solution as roots grown in 10 meq/l NaCl, if the split-medium or split-root techniques were used with comparatively low phosphorus concentration in the solution culture. All the experiments involving the split-medium and split-root techniques showed rather conclusively that bicarbonate had very little direct effect upon chlorosis or cytochrome oxidase activity when iron, phosphorus, bicarbonate, and calcium were not in direct contact with each other. However, at higher phosphorus levels, cytochrome oxidase activity was decreased in PI soybeans only in the presence of bicarbonate. This reduction in cytochrome oxidase was manifested whether enzyme activity was calculated on a protein or fresh weight basis. This indicates an interrelationship between phosphorus and bicarbonate on cytochrome oxidase activity.

In experiments using complete solution cultures (no split-root or split-medium) cytochrome oxidase activity of the roots was decreased with increases in the bicarbonate concentration and the resulting increase in soluble phosphorus in solution.

Cytochrome oxidase activity was not related to development of chlorosis in all cases. Enzyme activity varied with the phosphorus concentration in solution. The effect of bicarbonate on chlorosis is, in part, the interrelationship between bicarbonate, iron, phosphorus, and calcium in the growth medium. Bicarbonate per se, does not appear to be a direct cause of iron chlorosis or an inactivator of cytochrome oxidase at the concentrations used in these experiments. Further experiments are necessary to clarify the effect of phosphorus on cytochrome oxidase activity.

SUMMARY

PI–54619–5–1 (PI) and Hawkeye (HA) soybeans were grown using split-medium and split-root techniques to separate iron from the precipitating effects of bicarbonate and phosphorus. For comparative purposes, these were grown in complete solution cultures where iron, bicarbonate, and phosphorus were in direct contact with each other.

PI and HA soybeans did not develop chlorosis when grown using split-medium or split-root techniques with roots in 10 meq/l $NaHCO_3$ at comparatively low phosphorus concentration. In comparison, PI soybeans developed chlorosis in complete solution cultures when 2 meq/l $NaHCO_3$ was in solution. Cytochrome oxidase activity decreased with increasing bicarbonate concentration in complete solution cultures, but was dependent upon phosphorus concentration in the split-root experiments.

Bicarbonate in solution cultures increased the soluble phosphorus concentration in solution. Development of iron chlorosis was more related to phosphorus concentration in solution or that absorbed by the plant than by bicarbonate concentration in solution. Cytochrome oxidase activity was affected by the phosphorus concentration.

Bicarbonate does not appear to be a direct cause of iron chlorosis or directly inactivate cytochrome oxidase. An effect of phosphorus concentration on cytochrome oxidase activity was observed.

Literature Cited

1. Brown, J. C., O. R. Lunt, R. S. Holmes, and L. O. Tiffin. 1959. The bicarbonate ion as an indirect cause of iron chlorosis. Soil Sci. 88: 260–266.
2. Brown, J. C., R. S. Holmes, and L. O. Tiffin. 1959. Hypotheses concerning iron chlorosis. Soil Sci. Soc. Amer. Proc. 23: 231–234.
3. Brown, J. W., and C. H. Wadleigh. 1955. Influence of sodium carbonate on the growth and chlorosis of garden beets. Bot. Gaz. 116: 201–209.
4. Cooperstein, S. J., and A. Lazarow. 1952. A microspectrophotometric method for the determination of cytochrome oxidase. Jour. Biol. Chem. 189: 665–670.
5. DeKock, P. C. 1955. Iron nutrition in plants at high pH. Soil Sci. 79: 167–175.
6. Dickman, S. R., and R. H. Bray. 1940. Colorimetric determination of phosphate. Ind. Eng. Chem., Anal. Ed. 12: 665–668.
7. Doney, R., R. L. Smith, and H. Wiebe. 1960. The effects of various levels of HCO_3, P, and pH on the translocation of foliar applied Fe in plants. Soil Sci. 89: 269–275.
8. Goss, H. C. 1957. A comparison of effects of the bicarbonate ion with other anions on the uptake of some radioisotopes by *Phaseolus vulgaris* and *Hordeum vulgare* plants. Ph.D. Thesis, University of California, Los Angeles.
9. Greenwald, I. 1945. The effect of phosphate on the solubility of calcium carbonate and of bicarbonate on the solubility of calcium and magnesium phosphates. Jour. Biol. Chem. 161: 697–704.
10. Koening, R. A., and C. R. Johnson. 1942. Colorimetric determination of phosphorus in biological materials. Ind. Eng. Chem. Anal. Koening, ed. 14: 155–156.
11. Lindsay, W. L., and D. W. Thorne. 1954. Bicarbonate ion and oxygen level as related to chlorosis. Soil Sci. 77: 271–279.
12. Lowry, D. H., Nira J. Rosebrough, A. L. Farr, and Rose Randall. 1951. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275.
13. Miller, G. W., and H. J. Evans. 1956. The influence of salts on the activity of particulate cytochrome oxidase from roots of higher plants. Plant Physiol. 31: 357–364.
14. Miller, G. W., and H. J. Evans. 1956. Inhibition of plant cytochrome oxidase by bicarbonate. Nature 178: 974–976.
15. Miller, G. W., and D. W. Thorne. 1956. Effect of bicarbonate ion on the respiration of excised roots. Plant Physiol. 31: 151–155.
16. Porter, L. K., and D. W. Thorne. 1955. Interrelation of carbon dioxide and bicarbonate ions in causing plant chlorosis. Soil Sci. 79: 373–382.
17. Saywell, L. G., and B. B. Cunningham. 1937. Determination of iron, colorimetric o-phenanthroline method. Ind. Eng. Chem., Anal. Ed. 9: 67–69.
18. Thorne, D. W., F. H. Wann, and W. Robinson. 1951. Hypotheses concerning lime-induced chlorosis. Soil Sci. Soc. Amer. Proc. 15: 256–258.
19. Wadleigh, C. H., and J. W. Brown. 1952. The chemical status of bean plants afflicted with bicarbonate-induced chlorosis. Bot. Gaz. 113: 373–392.
20. Warnock, W. E. 1952. The iron content of some plants as influenced by conditions associated with lime-induced chlorosis. M.S. Thesis Utah State University, Logan, Utah.

OXIDATIVE ENZYMES OF SPINACH CHLOROPLASTS[1]

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In recent years, it has been found that isolated whole chloroplasts in light can carry out the processes of carbon dioxide fixation, photosynthetic phosphorylation, and photolysis of water (3). The presence in chloroplasts of the oxidative enzymes of the Krebs cycle, which are usually considered to be associated with the dark metabolism of the leaf, has apparently not been investigated extensively. An exception is the detailed study by James and Das (8) from which it was concluded that such oxidative enzymes are not present in purified chloroplasts. Arnon et al (3) were also unable to find these activities in chloroplasts. Several investigators have looked for cytochrome oxidase activity, but there is a lack of agreement among different workers as to its presence (13). Thus the question of the occurrence of oxidative enzymes in chloroplasts appears to be uncertain and deserving of further investigation.

Recent reports (11, 18) have shown that small particles, isolated from green spinach leaves by high speed centrifugation, are capable of oxidizing the acids of the Krebs cycle as well as reduced diphosphopyridine nucleotide (DPNH). A parallel efficient phosphorylation accompanies these oxidations. These observations suggested that the methods used to grind the leaves and to wash the active particles should be applied to the isolation of whole chloroplasts. Since glycerol had been used in the suspending medium in the method developed by James and Das (8), the effect of glycerol on the activity of both active particles and whole chloroplasts obtained from the same suspension was investigated. It has been found that the presence of glycerol in the suspending medium must be avoided if particles or chloroplasts possessing high oxidative enzyme activities are to be obtained.

Whole chloroplasts purified by repeated centrifugations in a suitable medium contain an active DPN-linked malic dehydrogenase, and rapid uptake of oxygen can be demonstrated with this substrate if a suitable hydrogen acceptor and a reagent which combines with carbonyl groups are provided. In addition, both glycolic acid oxidase and glyoxylic acid reductase have been shown to be present.

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MATERIALS AND METHODS

PREPARATION OF CHLOROPLASTS: Spinach was purchased locally and stored in a plastic bag at 5° C until used (not more than 4 days). The younger dark-green leaves were selected, the midribs were removed, and a weighed quantity of laminar tissue was washed in water and chilled on ice before being ground in the cold room.

Grinding was done with sand in a chilled mortar in two volumes of the grinding medium previously described for the preparation of active particles (11, 18). The medium consisted of 0.45 M sucrose, 0.05 M mannitol-borate buffer at pH 7.2, 0.05 M tris(hydroxymethyl)aminomethane chloride (Tris) buffer at pH 7.5, 0.03 M neutral potassium citrate, and 0.01 M sodium ethylenediamine tetraacetate (Versenate). The ground tissue was squeezed by hand through two layers of cheesecloth and centrifuged for 1 minute at 600 × G in a refrigerated centrifuge. The residue was discarded and the supernatant fluid was centrifuged at 1,000 × G for 12 minutes. The residue containing whole chloroplasts was gently suspended in a washing medium (1 ml per g of leaf) composed of 0.3 M sucrose, 2×10^{-4} M Versenate, and 0.05 M Tris buffer at pH 7.5 and again centrifuged for 12 minutes at 1,000 × G. The once-washed chloroplasts were again taken up in washing medium and the suspension was centrifuged for 1 minute at 200 × G to remove aggregated chloroplasts. After the number of washings indicated in the various experiments, the chloroplasts were suspended in 0.5 ml of washing medium per g of leaf.

When particles were to be isolated from the same suspension as the chloroplasts, the supernatant fluid decanted after the first period at 1,000 × G was centrifuged at 10,000 × G for 20 minutes. The residue was suspended and washed by centrifugation in the medium used for the purification of chloroplasts.

Total chlorophyll was determined in acetone extracts of the chloroplasts by measurement of the absorbancy at 645 mμ and 663 mμ (2). After the chloroplasts or particles were separated from the suspending medium by high speed centrifugation, total nitrogen was determined by Kjeldahl digestion and Nesslerization. Glyoxylate was determined colorimetrically as the 2,4-dinitrophenylhydrazone (19).

MANOMETRY: Oxygen uptake was measured by conventional techniques in 15 ml Warburg vessels in air. The vessels were shaken at 120 oscillations per minute, and light was excluded with a black cloth. All of the substrates and coenzymes used were of the highest grades commercially available. Reagent grade glycerol was purchased from the J. T. Baker Chemical Co. *N*-Methyl phenazonium sulfate (phenazine methosulfate) was obtained from the Aldrich Chemical Co. Sodium glyoxylate was prepared in this laboratory (12).

RESULTS

EFFECT OF GLYCEROL IN SUSPENDING MEDIUM ON OXIDATIVE ENZYME ACTIVITIES: In the experiments reported by James and Das (8), chloroplasts, but not particles, were isolated in a density gradient prepared from sucrose solution and glycerol. The final concentration of glycerol in which the chloroplasts were suspended was about 60 per cent (v/v). Chloroplasts isolated in this manner showed an active Hill reaction, but lacked cytochrome oxidase as well as the ability to oxidize malic acid and other acids of the Krebs cycle. Jagendorf (6) had previously suggested the use of still higher concentrations of glycerol in the suspending medium for the isolation of chloroplasts, but no oxidative enzyme activities were investigated with his preparations.

Experiments 1 and 2 (table I) show that the presence of 33 per cent glycerol (v/v) in the suspending medium is sufficient to reduce the succinate oxidase activity of particles markedly. The oxidation of malate and glycolate by twice-washed chloroplasts (expts 3 and 4) is likewise strongly inhibited by the addition of only 20 per cent glycerol (v/v) to the suspension. Although the mechanism by which this reduction in activity occurs is uncertain, it is evident that contact with moderately low concentrations of glycerol can damage oxidative enzyme activities of both particles and chloroplasts.

COMPARISON OF MALATE OXIDATION BY WHOLE CHLOROPLASTS AND PARTICLES: Isolated whole chloroplasts washed twice as described contain an active DPN-linked malic dehydrogenase which catalyzes the reversible oxidation of malate to oxaloacetate. This can be demonstrated directly with a dilute sus-

TABLE I

EFFECT OF GLYCEROL IN SUSPENDING MEDIUM ON OXIDATIVE ENZYME. ACTIVITIES OF ISOLATED PARTICLES AND CHLOROPLASTS

EXPERIMENT No.	PREPARATION	SUBSTRATE	OXYGEN UPTAKE IN μL/30 MIN*		
			CONTROL	GLYCEROL	% INHIBITION
1	Particles	Succinate	69.8	19.3	72
2	Particles	Succinate	86.1	25.2	71
3	Chloroplasts	Malate	31.7	16.9	47
		Glycolate	14.8	6.5	56
4	Chloroplasts	Malate	42.1	28.1	33
		Glycolate	23.5	12.0	49

* All figures are corrected for the slight activity without substrate.

The reaction mixture in experiments 1 and 2 contained the following components in Warburg vessels (18): sucrose, 300 μmoles; $MgSO_4$, 10 μmoles, succinate, 20 μmoles; potassium phosphate buffer at pH 7.0, 20 μmoles; adenosine triphosphate, 1 μmole; particles from 2.0 g of leaf tissue in 0.9 ml of washing medium; and water to make the final volume 2.0 ml. In the sidearm were placed glucose, 50 μmoles, and yeast hexokinase, 0.2 mg. The center well contained 0.2 ml of 5 *N* KOH. The reactions were carried out at 30° C. After the first centrifugation at 10,000 × G (18), particles were suspended in washing medium and two samples of 2 ml each were taken. Glycerol, 0.6 ml, was added with stirring to one sample to make the concentration 30 % (v/v) and then 10 ml of washing medium was added to each. The suspensions were then centrifuged for 20 minutes at 10,000 × G and each sample finally taken up in 2.5 ml of washing medium.

The reaction mixture for malate oxidation in experiments 3 and 4 consisted of the following components in Warburg vessels: Tris(hydroxymethyl)aminomethane buffer (Tris) at pH 7.0, 100 μmoles; potassium phosphate at pH 7.0, 10 μmoles; $MgSO_4$, 10 μmoles; sucrose, 300 μmoles; potassium L-malate, 40 μmoles; DPN, 0.45 μmole; sodium cyanide, 20 μmoles; chloroplasts representing 2.0 g of leaf tissue suspended in 0.9 ml of washing medium; and water to make the final volume 2.0 ml. The sidearm contained 1 mg of *N*-methyl phenazonium sulfate, and the center well 0.2 ml of 1 *N* sodium cyanide. The final pH of the reaction mixture was about 7.5. *N*-methyl phenazonium sulfate was added at zero time after temperature equilibration for 5 minutes at 30° C.

In experiments 3 and 4, glycolate oxidation was demonstrated in a manner similar to that used for oxygen uptake with malate. Tris buffer at pH 7.5 was used, and DPN, cyanide, and hydrogen carrier were omitted. Potassium glycolate, 20 μmoles, was the substrate, and 5 *N* KOH was added to the center well.

In experiments 3 and 4, the crude chloroplast suspension was obtained as described in the text, and divided into two equal samples. Glycerol (20 % v/v) was added with stirring to one portion, and a corresponding volume of water was added to the control suspension. The chloroplasts were then washed twice and taken up in washing medium as usual. In experiment 3, there was 0.13 mg of chlorophyll per vessel in the control and 0.10 mg of chlorophyll in the glycerol-treated chloroplasts. In experiment 4, the chlorophyll concentration was 0.11 mg and 0.09 mg per vessel, respectively.

pension of chloroplasts by observing the formation of DPNH from DPN at 340 mμ in the presence of malate. The DPNH can subsequently be oxidized by addition of a low concentration of oxaloacetate to shift the equilibrium (fig 1). When a small amount of cyanide is added at the start to combine with the oxaloacetate formed, more DPNH is produced as expected.

Oxygen uptake by twice-washed chloroplasts can also be readily demonstrated with malate as the substrate (table II). In addition to DPN, a suitable hydrogen acceptor such as *N*-methyl phenazonium sulfate (4), and a carbonyl-binding reagent such as cyanide or hydroxylamine must be provided. As has been frequently shown in other systems (5), the equilibrium of the malic dehydrogenase reaction is unfavorable in the forward direction, and it is necessary to remove the oxaloacetate produced in order to obtain rapid rates of oxygen uptake. TPN does not replace DPN, however, indicating that there is little malic enzyme activity in these preparations. Methylene blue (0.5 mg per vessel) can be substituted for *N*-methyl phenazonium sulfate, although the rates of oxygen uptake are not quite as high. Since an autooxidizable hydrogen acceptor must be added, it is apparent that these preparations of chloroplasts, unlike particles (7, 18), are unable to transfer hydrogen rapidly from malate directly to oxygen.

Particles from spinach leaves provided only with DPN rapidly oxidize malate (18). The requirements

TABLE II

REQUIREMENTS FOR MALATE OXIDATION BY TWICE-WASHED CHLOROPLASTS

EXPERIMENT No.	SYSTEM	OXYGEN UPTAKE IN μL/30 MIN
1	Complete	42.9
	No DPN	8.4
	No cyanide	7.9
	No *N*-methyl phenazonium sulfate	0
	No malate	0
	Complete, hydroxylamine instead of cyanide	38.6
2	Complete	37.8
	No DPN	0
	No malate	4.8
	Complete, TPN instead of DPN	7.4

The complete system for malate oxidation contained the components shown in table I in Warburg vessels. Twice-washed chloroplasts containing 0.15 mg of chlorophyll per vessel in experiment 1 and 0.23 mg of chlorophyll in experiment 2 were used. In experiment 1, when neutralized hydroxyamine hydrochloride, 50 μmoles, was used, KOH was placed in the center well. In experiment 2, where indicated, 0.45 μmole of TPN was substituted for the same concentration of DPN.

The reaction rates were measured for 30 minutes at 30° C after tipping in *N*-methyl phenazonium sulfate from the sidearm.

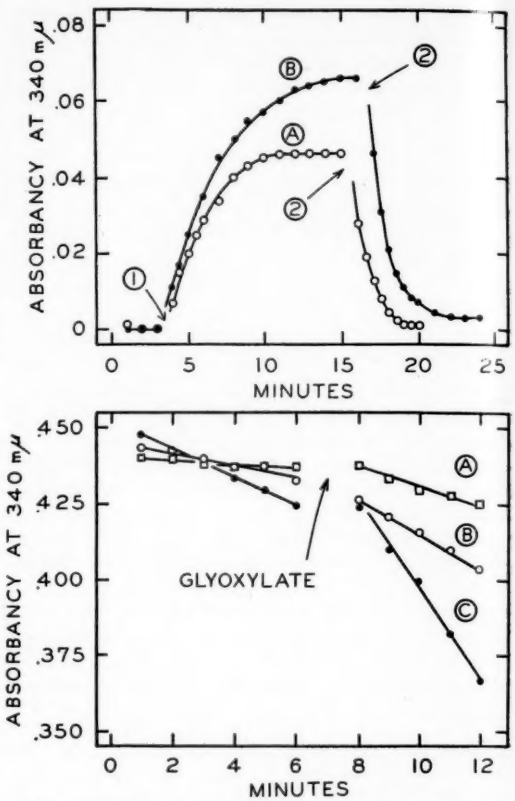

FIG. 1. Direct demonstration of malic dehydrogenase in twice-washed chloroplasts. Curve A shows results when 200 μmoles of Tris buffer at pH 7.5, 700 μmoles of sucrose, and water to make the final volume 3.0 ml were added to cuvettes of 1.0 cm light path. Chloroplasts, 0.1 ml, containing 0.018 mg of chlorophyll were added, and at zero time 0.9 μmole of DPN was placed in the experimental cuvette. Readings were made at 340 mμ in the Beckman spectrophotometer against a cuvette containing all components except DPN. At arrow 1, 60 μmoles of potassium L-malate was added, and at arrow 2, one μmole of potassium oxaloacetate was also added to each cuvette. For the data shown in curve B, the treatment was the same except that 0.3 μmole of sodium cyanide was present before the DPN was added at zero time. The reactions were carried out at about 25° C.

FIG. 2. Glyoxylic acid reductase activity of twice-washed chloroplasts. To cuvettes of 1.0 cm light path were added 200 μmoles of potassium phosphate buffer at pH 7.0, 700 μmoles of sucrose, and water to make the final volume 3.0 ml. A twice-washed chloroplast suspension containing 0.0075 mg of chlorophyll (curve A), 0.015 mg of chlorophyll (curve B), and 0.030 mg of chlorophyll (curve C) was added to each cuvette, and at zero time 0.21 μmole of DPNH was added to the experimental cuvette. Readings were taken at 25° C against a sample containing all of the components except DPNH. At the arrow, 30 μmoles of sodium glyoxylate was added to each cuvette.

TABLE III

REQUIREMENTS FOR MALATE OXIDATION BY CHLOROPLASTS AND PARTICLES

SYSTEM	μL OXYGEN UPTAKE IN 30 MIN/MG TOTAL N*			
	EXPERIMENT 1		EXPERIMENT 2	
	CHLOROPLASTS	PARTICLES	CHLOROPLASTS	PARTICLES
Complete	231	178	126	165
No *N*-methyl phenazonium sulfate and no carbonyl-binding reagent	0	153	0	44

* Corrected for the slight activity without substrate.

Chloroplasts and particles were isolated from the same homogenate in each experiment and washed once. The components added to the Warburg vessels were the same as those described in table I for malate oxidation.

In experiment 1, 50 μmoles of hydroxylamine was added to react with the oxaloacetate formed; the total N per vessel was 0.19 mg for the chloroplasts and 0.45 mg for the particles; the ratio of the mg of total N to the mg of chlorophyll in the chloroplasts used was 1.7.

In experiment 2, 20 μmoles of cyanide was used to react with the oxaloacetate; there was 0.40 mg of total N per vessel in the chloroplasts and 0.53 mg in the particles; the ratio of the mg of total N to the mg of chlorophyll in the chloroplast preparation was 1.8. In the presence of cyanide, there was no oxygen uptake with particles for 10 minutes before the *N*-methyl phenazonium sulfate was tipped in at zero time, while particles without cyanide absorbed oxygen throughout the experiment at a constant rate.

for malate oxidation by chloroplasts and particles isolated from the same initial suspension and washed in an identical manner were compared in terms of total N content (table III). It is apparent that such particles contain a complete system for directly and rapidly transferring hydrogen from DPNH to oxygen, while the chloroplasts were deficient in this activity. A suitable hydrogen carrier and a carbonyl-trapping reagent are required by chloroplasts if the activity is to be demonstrated.

GLYCOLIC ACID OXIDASE AND GLYOXYLIC ACID REDUCTASE ACTIVITY OF CHLOROPLASTS: Tolbert and Cohan (14) have reported that some glycolic acid oxidase activity is associated with chloroplasts and chloroplast fragments isolated from barley leaves. Addition of glycolate to washed whole chloroplasts has always been observed to give rise to a marked increase in oxygen uptake without further supplements of cofactors (table I, table IV). In an experiment designed to test the stoichiometry of the reaction, twice-washed chloroplasts provided with glycolate took up 2.9 μatoms of oxygen and produced 3.2 μmoles of glyoxylate. This suggests that a sufficient excess of catalase was present (19), and that the reaction is consistent with the equation:

$$\text{Glycolate} + 0.5\ O_2 \rightarrow \text{glyoxylate} + H_2O$$

As is shown in table IV, suspension and centrifugation of whole chloroplasts as many as four times does not appreciably alter the glycolic acid oxidase activity expressed in terms of chlorophyll concentration, thus supporting the view that this enzyme is present in the chloroplast.

Glyoxylic acid reductase activity (15) was demonstrated spectrophotometrically in dilute suspensions of chloroplasts by measuring the rate of oxidation of

TABLE IV

EFFECT OF SUCCESSIVE WASHING OF CHLOROPLASTS ON OXIDATION OF MALATE AND GLYCOLATE

EXPERIMENT No.	SUBSTRATE	No. OF WASHINGS	MG CHLOROPHYLL/μL VESSEL	OXYGEN/30 MIN MG CHLOROPHYLL
1	Malate	1	0.11	391
	Malate	2	0.089	549
	Glycolate	1	0.11	151
	Glycolate	2	0.089	237
2	Malate	2	0.17	202
	Malate	4	0.15	217
	Glycolate	2	0.17	108
	Glycolate	4	0.15	86

Chloroplasts were washed by successive centrifugations at 1,000 × G for 12 minutes followed by suspension in a volume of washing medium equal to the initial fresh weight of the leaves. The components in the Warburg vessels were the same as those shown in table I. In experiment 1, 50 μmoles of hydroxylamine were added to serve as the carbonyl-trapping agent for malate oxidation, and in experiment 2 sodium cyanide (20 μmoles) served this function.

In experiment 1, the ratio of mg total N to mg of chlorophyll was 1.7 for once-washed chloroplasts and 1.9 for twice-washed chloroplasts.

DPNH on addition of glyoxylate (fig 2). After correction for the DPNH oxidase present, the glyoxylic acid reductase activity was proportional to the chloroplast concentration over a four-fold range, and was calculated to be equivalent to the reduction of 3.4 μmoles of glyoxylate per mg of chlorophyll in 30 minutes at 25° C. This same preparation of chloroplasts oxidized 7.4 μmoles of glycolate in 30 minutes per mg of chlorophyll at 30° C. Thus the activities of these two enzymes in chloroplasts are similar in magnitude.

Oxidation of Other Substrates by Whole Chloroplasts: Jagendorf (7) has found that chloroplasts can oxidize DPNH directly at a slow rate, 0.1 to 0.4 μmole per mg of chlorophyll per hour. In some of his experiments, a medium containing 50 per cent glycerol (v/v) was apparently used to prepare the chloroplasts. Twice-washed chloroplasts prepared by the procedure described under Methods also contain DPNH oxidase (fig 2), with an activity of about 4.6 μmoles per hour per mg of chlorophyll.

Under conditions in which whole chloroplasts rapidly oxidize malate (table II), there was only a negligible oxygen uptake when succinate or pyruvate (and a catalytic amount of malate) was provided. Addition of yeast cofactors was also without effect. Occasionally, in some experiments, a stimulation of oxygen uptake with succinate as substrate was observed, but the activity was low compared with the rate of oxidation of malate.

Since the possibility exists that some substrates cannot enter the chloroplasts, an acetone powder was prepared from whole chloroplasts. After extraction of the powder with 0.1 M Tris buffer at pH 8.3, the extract was tested for oxidative enzyme activity in Warburg vessels containing DPN and *N*-methyl phenazonium sulfate. Under these conditions, with a final volume of 2.0 ml, in 50 minutes the addition of 40 μmoles of malate brought about an oxygen uptake of 72 μl; with 40 μmoles of fumarate, 8.4 μl of oxygen were taken up; with 40 μmoles of succinate, 4.3 μl; and with 40 μmoles of pyruvate, 3.0 μl. Of the activities tested, malic dehydrogenase is thus the only enzyme thoroughly established to be present in significant amounts in the acetone powder of whole chloroplasts.

Discussion

Microscopic examination of the preparations of particles revealed a heterogeneous suspension consisting of chloroplast fragments and other small cellular inclusions, presumably mitochondria. The washed chloroplasts obtained by the methods described appear to be intact. From the evidence presented, it is clear that no biochemically active particles are present with the chloroplasts since malate is not oxidized by the chloroplast preparation without the addition of a hydrogen acceptor, while particles treated in the same manner as the chloroplasts oxidize malate without an extraneous hydrogen acceptor. The integrity of the chloroplasts is further supported by the observation that the ratio of total N to chlorophyll does not change appreciably during successive washings (table IV), and that this ratio is constant in different preparations (table III).

It is not unusual to find that specific enzymes of otherwise intact chloroplasts may be selectively damaged. For example, Allen et al (1) have reported that treatment of isolated chloroplasts with water has no effect on the photolysis or photosynthetic phosphorylation activities, but completely abolishes the ability of the chloroplasts to fix CO_2. Likewise, the experiments described here show that glycerol in high concentrations greatly diminishes the activity of some oxidative enzymes of chloroplasts, while James and Das (8) have reported that it has no effect on the Hill reaction. The deleterious action of glycerol may arise from its ability to extract enzymes or to inactivate them. When 12.5 per cent glycerol (v/v) was added to particles obtained as described, and permitted to remain in contact (table I) for 15 and 30 minute periods before dilution and centrifugation, the rate of the oxidation of succinate was lowered 40 and 60 per cent, respectively, compared with untreated controls. This experiment, together with the observation (table I) that several of the different activities are lowered to approximately the same extent by exposure to glycerol, makes it likely that glycerol extracts rather than inactivates some enzymes.

Chloroplasts contain an active cytochrome c reductase which can serve to oxidize either DPNH or TPNH (9). It thus seems probable that the failure to demonstrate oxygen uptake with chloroplasts on addition of only malate and DPN is attributable to a lack of cytochrome oxidase in the preparations, since the addition of cytochrome c (1 mg/vessel) with or without hydroxylamine did not replace the requirement for *N*-methyl phenazonium sulfate. This observation also accounts for the previous failure of Arnon et al (3) to find oxygen uptake with malate as substrate, since these workers added no exogenous hydrogen acceptor.

Nieman and Vennesland (10) have commented on the great variability of the dark cytochrome oxidase activity among the different chloroplast preparations used in their experiments. The question whether the occasional presence of this activity represents a contamination, or its absence indicates that it has been destroyed during preparation remains unsettled. However, if absence of cytochrome oxidase indicates an inactivation during isolation of the chloroplasts, this loss of activity must occur under conditions in which the activity of particles is retained (table III). It is possible that the age of the spinach leaves used, or some other physiological factor determines whether cytochrome oxidase can be demonstrated in chloroplasts.

In addition to the evidence already presented for the presence of some oxidative enzymes in whole chloroplasts, it has been shown that malic dehydrogenase and glycolic acid oxidase are not removed by repeated suspension and centrifugation in fresh washing medium (table IV). Moreover, the activities

of these two enzymes have been found to be approximately proportional to the chloroplast concentration over a four-fold range. As has recently been demonstrated, glycolic acid oxidase functions exclusively in sunlight (16, 17); hence it is not surprising that this activity should reside in the chloroplast. The high activity of this enzyme in aqueous extracts of leaves probably reflects the ease with which some chloroplast proteins can be extracted, and suggests that much of the soluble protein of leaves may originate in the chloroplast. The glycolic acid oxidase activity previously found in particles (18) undoubtedly arises from chloroplast fragments present in these preparations. Zucker (20) has recently reported that the activities of malic dehydrogenase, glycolic acid oxidase, and glyoxylic acid reductase increased in proportion to the increase in soluble leaf protein, when extracts of tobacco leaves from plants grown in sunlight were compared with extracts from plants grown in the shade. This finding suggests that the increase in the leaf protein represents an increase in the chloroplast protein.

The failure to demonstrate in whole chloroplasts enzymes of the Krebs cycle other than malic dehydrogenase does not necessarily mean that these activities are missing. Perhaps for reasons which are not now apparent, some enzymes which are normally present in chloroplasts have been extracted or inactivated. The activities that have been demonstrated may accordingly represent only a part of the oxidative enzymes present.

Summary

Whole chloroplasts have been prepared from spinach leaves with use of the media which were previously found to be suitable for the isolation of particles possessing high rates of oxidative enzyme activity. It was found that the presence of glycerol in the suspending medium has deleterious effects on the oxidative enzymes of both particles and chloroplasts. Whole chloroplasts contained an active DPN-linked malic dehydrogenase, but, unlike particles, required addition of an extraneous hydrogen acceptor, such as *N*-methyl phenazonium sulfate, and a carbonyl-binding reagent, such as cyanide, in order to demonstrate high rates of oxygen uptake. Besides malic dehydrogenase, glycolic acid oxidase, and glyoxylic acid reductase have also been found to be present in repeatedly washed chloroplasts. Other enzymes of the Krebs cycle could not be demonstrated in these chloroplast preparations.

Acknowledgments

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Literature Cited

1. Allen, M. B., D. I. Arnon, J. B. Capindale, F. R. Whatley, and L. J. Durham. 1955. Photosynthesis by isolated chloroplasts. III. Evidence for complete photosynthesis. Jour. Amer. Chem. Soc. 77: 4149–4155.
2. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
3. Arnon, D. I., M. B. Allen, and F. R. Whatley. 1956. Photosynthesis by isolated chloroplasts. IV. General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta 20: 449–462.
4. Dickens, F. and H. McIlvain. 1938. Phenazine compounds as carriers in the hexosemonophosphate system. Biochem. Jour. 32: 1615–1625.
5. Green, D. E. and J. Brosteaux. 1936. The lactic dehydrogenase of animal tissues. Biochem. Jour. 30: 1489–1508.
6. Jagendorf, A. T. 1955. Purification of chloroplasts by a density technique. Plant Physiol. 30: 138–143.
7. Jagendorf, A. T. 1956. Oxidation and reduction of pyridine nucleotides by purified chloroplasts. Arch. Biochem. Biophys. 62: 141–150.
8. James, W. O. and V. S. R. Das. 1957. The organization of respiration in chlorophyllous cells. New Phytologist 56: 325–343.
9. Marrè, E. and O. Servettaz. 1958. TPNH-cytochrome c reductase of chloroplasts and its role in photosynthetic phosphorylation. Arch. Biochem. Biophys. 75: 309–323.
10. Nieman, R. H. and B. Vennesland. 1959. Photoreduction and photooxidation of cytochrome c by spinach chloroplast preparations. Plant Physiol. 34: 255–262.
11. Ohmura, T. 1955. Oxidative phosphorylation by a particulate fraction from green leaves. Arch. Biochem. Biophys. 57: 187–194.
12. Radin, N. S. and D. E. Metzler. 1955. Sodium glyoxylate monohydrate. Biochem. Preparations 4: 60–62.
13. Sissakian, N. M. 1958. Enzymology of the plastids. Advances in Enzymol. 20: 201–236.
14. Tolbert, N. E. and M. S. Cohan. 1953. Products formed from glycolic acid in plants. Jour. Biol. Chem. 204: 649–654.
15. Zelitch, I. 1955. The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. Jour. Biol. Chem. 216: 553–575.
16. Zelitch, I. 1958. The role of glycolic acid oxidase in the respiration of leaves. Jour. Biol. Chem. 233: 1299–1303.
17. Zelitch, I. 1959. The relationship of glycolic acid to respiration and photosynthesis in tobacco leaves. Jour. Biol. Chem. 234: 3077–3081.
18. Zelitch, I. and G. A. Barber. 1960. Oxidative phosphorylation and glycolate oxidation by particles from spinach leaves. Plant Physiol. 35: 205–209.
19. Zelitch, I. and Ochoa, S. 1953. Oxidation and reduction of glycolic and glyoxylic acids in plants. I. Glycolic acid oxidase. Jour. Biol. Chem. 201: 707–718.
20. Zucker, M. 1959. Alteration of the protein components and other constituents in the tobacco leaf by shading. Plant Physiol. 34: 563–569.

PHYSIOLOGICAL STUDIES OF CHILLING INJURY IN CITRUS FRUITS[1, 2]

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Chilling caused by temperatures below 10° C but above the freezing point of the tissue have been reported to cause physiological upsets and even death to certain plant tissues. The effects of chilling on sensitive tissues have been described and reviewed (3, 8, 9, 14, 16). However, the physiological and metabolic aspects of this phenomenon have received limited attention (8, 11, 12, 13). The respiratory response has been used as a criterion of the physiological effects of chilling injury. Stimulation of the respiratory rate at chilling temperatures has been reported for cucumbers (8) and for sweet potatoes (12). The influence of a previous exposure to chilling temperatures on the respiratory rate and trends at non-chilling temperatures has been investigated (1, 4, 5, 6, 8, 10, 17, 18). Most, but not all of these studies showed the initial rates of CO_2 evolution, after transfer to the warmer temperature, rising to a maximum well above the value normally expected and then slowly falling back to the expected value. This stimulation in CO_2 output, with two exceptions (8, 17) has not been associated with chilling injury sustained by the material. The methods for determining CO_2 production available to these investigators required sampling and/or equilibrium periods of 6 to 48 hours. Thus, the rates obtained were averages for the period sampled. Hence, the initial and peak rates may have been obscured. It appeared, therefore, that with the new methods available a better understanding of the processes leading to chilling injury might be gained by critically investigating the gas exchange and internal atmosphere during the period immediately after transfer from a chilling to a non-chilling temperature.

This report concerning the physiological responses of citrus fruits to chilling describes A. the variations in CO_2 production of citrus fruits produced by chilling from determinations made continuously 5 minutes after transfer from chilling to non-chilling temperatures, B. the results of analysis of the internal atmosphere and total CO_2 content of these fruits both during and after the chilling exposure, and C. the variations in the rate of O_2 uptake of immature orange, lemon, and lime fruit at 20° C after a chilling exposure.

MATERIALS AND METHODS

The orange fruit, *Citrus sinensis* (Linn.) Osbeck, used in this study was selected from freshly harvested fruit in commercial packing houses or in Citrus Experiment Station groves. The fruit was held at 20° C, usually less than 2 days, until the beginning of the experimental period. During the experimental period the fruit was held in open containers at 0°, 10°, and 20° C.

The rate of CO_2 production was determined by a Liston-Becker Infra-red Analyzer (Model 15) connected to a Leeds and Northrup strip recorder. With this instrument it was possible to obtain continuous readings on the fruits, beginning immediately after transfer to 20° C, in contrast to previous absorption methods in which the sampling period ranged from 6 to 48 hours. The free volume in the chambers was about 250 ml with metered airflow of 100 ml per minute. Equilibrium within the chamber was quickly established and maintained. Rates were calculated at hourly intervals, except where a change in slope occurred; then half-hour intervals were used. The coefficients of variability for the average rates of different fruits receiving the same treatment were less than 5%, thus indicating good agreement between fruits. The instrument was adjusted to the range of 150 to 1,300 ppm CO_2. Calibration curves were made with known CO_2 concentrations prepared by mixing 0.95% CO_2 with pure nitrogen, using calibrated capillaries. The individual fruits were placed in wide-mouthed Mason jars, each fitted with a No. 14 rubber stopper. To determine the rate of temperature change after transfer to 20° C, thermocouples were placed at various depths in the fruits in the respiratory chambers. Under the existing conditions, temperature equilibrium of the entire fruit was attained within 5 hours. Thus the respiratory rates in the beginning are composites of the increasing rate due to effects of temperature and metabolic activity, moderated by diffusion. In some experiments, the effect of temperature was eliminated by warming the fruits in a well-agitated water bath for 1 hour, to establish temperature equilibrium throughout the fruits before they were placed in the respiratory chambers.

The oxygen uptake was measured manometrically in the Warburg apparatus at 20° C. Immature fruits weighing from 11 to 14 g were used. These fruits were harvested from Experiment Station groves. Determinations were made on freshly harvested fruits and after exposure for various periods to 0°, 10°, and 20° C. During the exposure period the fruits were held in glass chambers aerated with humidified air. The fruits were brought to temperature equilibrium at 20° C in an agitated water bath before being placed in the Warburg flasks.

The internal atmosphere and total CO_2 content of the fruits were followed at 20° C after exposure to 0° and 10° C by methods reported previously (7). Briefly, the methods may be described as follows: A. A vacuum was used to remove the internal atmosphere, which was then analyzed for CO_2 and O_2 in an Orsat-type gas-analysis apparatus. B. The

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total CO_2 content was determined by grinding the fruit in alkali and acidifying it. The CO_2 was aerated into alkali and determined by titration. Both methods ensure against loss of gas from the internal atmosphere of citrus fruit, which is normally under positive pressure. All treatments, respiratory rates, and internal atmospheres were replicated at least four times.

Results

The milligrams of carbon dioxide (CO_2) per kilogram per hour produced by mature navel orange fruits held at 20° C after exposure to various temperature (0°, 10°, 20°, and 30° C) for various periods are shown in figure 1. Little difference in the rate of CO_2 production of fruits held at 20° C for 4, 10, and 19 days was noted; therefore, the values of all fruits observed were averaged. This average serves as a control to appraise the effect of the other treatments. The rate of CO_2 output of fruits previously held at 30° C decreased with time to essentially that of fruits held at 20° C. The fruits that had been cooled rapidly in water and held for 4 hours in air at 0° and 10° C showed slightly higher rates of CO_2 production than control fruits held at 20° C. Similar responses to hydrocooling have been reported (6). Exposure of fruit to temperatures of 0° and 10° C for 4, 10, and 19 days increased CO_2 production at 20° C. Peak rates were attained 8 to 10 hours after transfer of the fruit to a temperature of 20° C. The stimulatory effect increased with increasing exposure time, and fruit held at 0° C showed a greater response than that held at 10° C. The effect persisted for 1 to 4 days, the time depending on the length of the exposure period.

The milligrams of CO_2 produced per kilogram of fruit in excess of the amount produced by the fruit held at 20° C are shown in table I part a. Note that this amount increased with increased exposure and that fruit held at 0° C produced more CO_2 than fruit held at 10° C. Experiments conducted with Valencia orange fruits produced essentially the same results.

The data presented in figure 1 illustrate in the initial phase the interaction of increasing rate of respiration due A. To temperature change and B. To the effect of exposure to low temperature. Both are moderated by diffusion out of the fruit. To eliminate the effect of temperature change, navel oranges were held for 4 days at 0° and 20° C and then warmed rapidly in a 20° C, well-agitated water bath for 1 hour before being placed in the respiratory chambers. Little effect was observed for fruits from 20° C. Fruits previously held at 0° C had initial rates of CO_2 production of about 60 mg CO_2/kg/hr, which decreased with time and after about eight hours closely paralleled that of the samples put directly into the chambers (fig 1, 4 days at 0° C and 20° C).

An evaluation of the rate of O_2 uptake by immature orange, lemon, and lime fruits was made. Determinations were made at 20° C on fruits held 6 and 12 days at 0°, 10°, and 20° C. To avoid the injury effect of harvesting, fruits held for 1 day at 20° C were used for comparison instead of freshly harvested fruits. The results for immature orange fruits held for 12 days at 20° C are presented in figure 2. After 12 days at 20° C, the rate of O_2 uptake was lower than that of fruits held 1 day at 20° C. Fruits held at 10° C had higher rates of uptake than fruits held 1 day at 20° C. Holding at 0° gave the highest rates.

The curves for rates of O_2 uptake by immature fruits have the same characteristic shape as the curves for CO_2 output by mature fruits warmed rapidly to 20° C before determinations were begun; that is, the rate was high initially and decreased with time. The response of fruits held 6 days at 0°, 10° and 20° C was similar at 20° C to that of fruits held 12 days at these temperatures. Comparable results were obtained with immature lemons and limes.

Preliminary observations on CO_2 production at a temperature of 20° C after storage at 0°, 5°, 10°, and 20° C, were made on carrots, sweet potatoes, and cucumbers. The peak rates of CO_2 production after transfer to 20° are presented in table II. Carrot roots, which are not considered susceptible to chilling injury, and which had been held at 0° and 10° C, showed an increasing rate of CO_2 production as they warmed to that of the roots which had been held at 20° C, and closely followed it without an observed stimulation. Similar results were reported in 1936 by Appleman and Smith (1). Sweet potato roots and cucumber fruits which are susceptible to chilling injury showed stimulation (table II). Stimulation above that of the same material held at 20° C persisted from 4 to 9 days. The sustained stimulatory effect found here on cucumber fruits is in agreement with that of a previous report (8), but in that report determinations were not made until 24 hours after

Table I

Part a. CO_2 Evolved at 20° C by Fruits Held 4, 10, and 19 Days at 0° and 10° C in Excess of Amount of CO_2 Evolved by Fruits Previously Held at 20° C

Days held at temperature	Mg CO_2/kg fruit	
	Previous holding temperature	
	10° C	0° C
4	350	900
10	600	1,400
19	20,000	30,000

Part b. Total CO_2 Content of Orange Fruit when Held 6 Days at 20°, 10° and 0° C, and After 3 Additional Days at 20° C

	Mg CO_2/kg fruit		
	holding temperature		
	20° C	10° C	0° C
After 6 days at temperature	210	186	126
6 Days at temperature + 63 hrs at 20° C	212	232	246

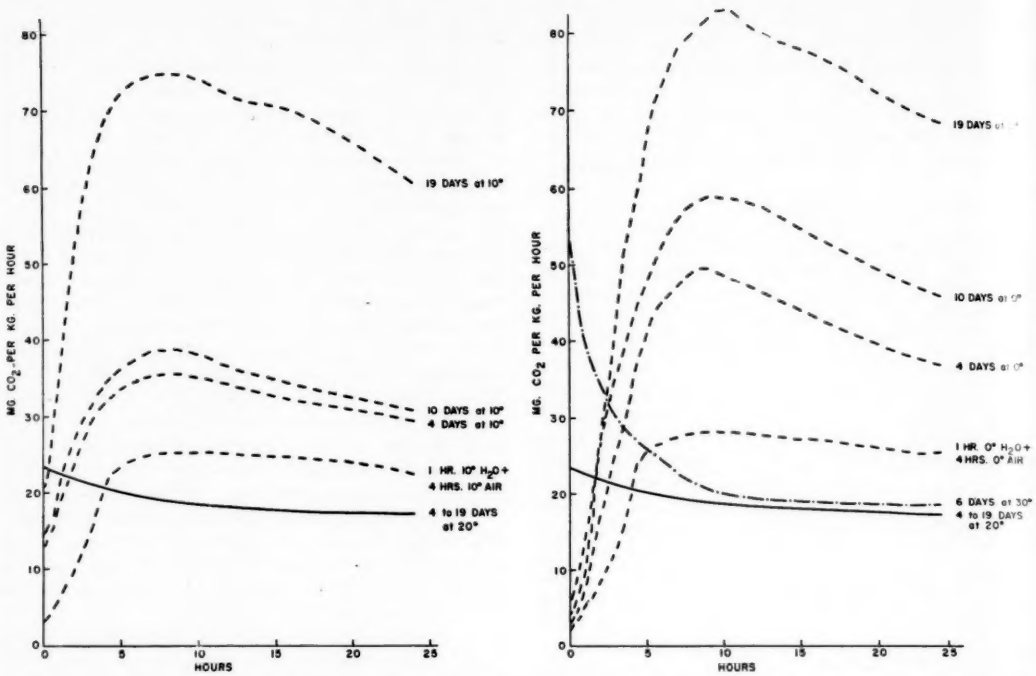

MG. CO_2 PER KG. PER HOUR
HOURS
19 DAYS at 10°
10 DAYS at 10°
4 DAYS at 10°
1 HR. 10° H_2O + 4 HRS. 10° AIR
4 to 19 DAYS at 20°
MG. CO_2 PER KG. PER HOUR
HOURS
19 DAYS at
10 DAYS at
4 DAYS at
1 HR. 0° H_2O + 4 HRS. 0° AIR
6 DAYS at 30°
4 to 19 DAYS at 20°

FIG. 1. Carbon dioxide production by mature navel orange fruits at 20° C after various exposures to 0°, 10°, 20° and 30° C.

transfer, and the peak rates found in the present study were therefore not observed. Other products such as avocados, bananas, and tomatoes were observed, but the data are difficult to interpret because the climacteric rise in respiration characteristic of these fruits was differentially hastened or suppressed by the different holding temperatures and became integrated with the effect of temperature on CO_2 production.

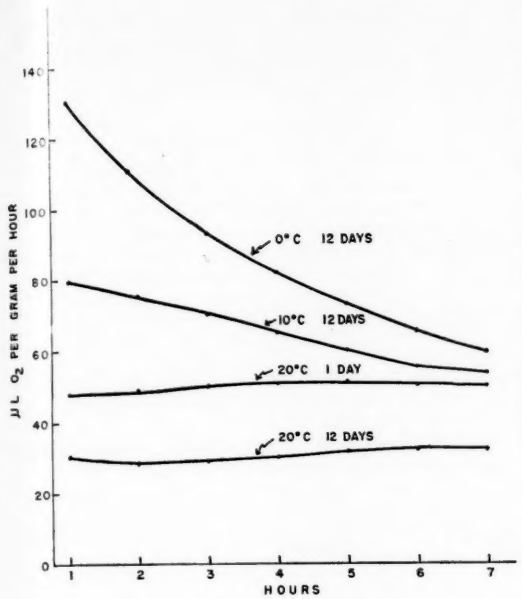
Direct evidence relative to the contribution of physical processes to the increase in evolved CO_2 was obtained by determining the composition of the internal atmosphere and the total CO_2 content of orange fruits. Determinations were made on fruits that had been held 6 days at 20°, 10°, and 0° C and also on fruits that had been held 6 days at 10° and 0° C, 1, 5, 15, and 63 hours after transfer to 20° C.

Figure 3 shows the percentages of CO_2 and O_2 in the internal atmosphere of orange fruits 0, 1, 5, 15, and 63 hours after transfer to 20° C following a 6-day exposure to temperatures of 0° and 10° C. Confirming the previous report (7), the initial CO_2 percentage increased as the holding temperature increased. The percentage of CO_2 in fruits transferred from 0° and 10° C increased for the first 15 hours after transfer to 20° C and then decreased slightly. The percentage of O_2 was high initially, but decreased to a minimum 15 hours after transfer and then increased slightly. The highest CO_2 and the lowest O_2 percentage values were observed 15 hours after transfer.

TABLE II

PEAK RATES OF CO_2 PRODUCTION AT 20° C BY CARROTS, SWEET POTATOES, AND CUCUMBERS AFTER EXPOSURE TO VARIOUS TEMPERATURES

VEGETABLE MATERIAL	MG CO_2/KG/HR AT VARIOUS TEMPS AND HOLDING PERIODS					
	20° C FOR 6 DAYS	10° C FOR 6 DAYS	5° C FOR 6 DAYS	5° C FOR 13 DAYS	5° C FOR 23 DAYS	0° C FOR 6 DAYS
Carrot	30	31	...	...	...	34
Sweet potato	45	...	90	100	160	...
Cucumber	25	65	...	...	...	160



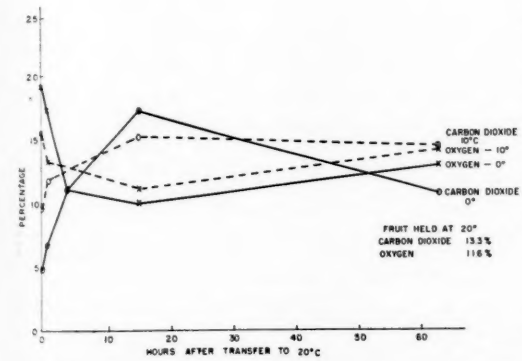


FIG. 2 (*upper*). Oxygen uptake by immature orange fruits at 20° C after 1 day at 20° C and 12 days at 0°, 10° and 20° C.

FIG. 3 (*lower*). Percentage carbon dioxide and oxygen in the internal atmosphere of orange fruits 0, 1, 5, 15, and 63 hours after transfer to 20° C following a 6-day exposure to 0° and 10° C. Internal atmosphere was obtained by the vacuum method (7).

Higher partial pressure of CO_2 in fruits at higher temperatures would be expected in view of the more rapid rate of CO_2 production by the fruit at higher temperatures. Restriction of diffusion offered by the fruit tissue and rind would cause a higher CO_2 diffusion gradient and maintain a higher partial pressure of CO_2 inside the fruit. This in turn would lead to an increase in the total CO_2 content of the fruit. Increase in the partial pressure of CO_2 in the internal atmosphere of the fruit occasioned by the transfer from a low to a high temperature constitutes evidence, although indirect, that physical processes do not contribute to the increase in CO_2 evolution. The concomitant determinations of the total CO_2 content definitely show that physical processes are not involved. Table I, part b presents the total CO_2 content of orange fruits held 6 days at 20°, 10°, and 0° C and of fruits held 6 days at 10° and 0° C plus 63 hours at 20° C. The total CO_2 content of orange fruits decreased as the holding temperature decreased (table Ib). After transfer to 20° C from 10° and 0° C, the total CO_2 content increased and remained high. Plots of total CO_2 content 1, 5, 15, and 63 hours after transfer to 20° C are similar to the percentage CO_2 shown in figure 3, except that the difference between 15 and 63 hours for the fruits previously held at 0° C was small. These data indicate that after transfer from 0° and 10° to 20° C a small part of the metabolically produced CO_2 remained in the fruit instead of being released to contribute to the flush of CO_2 evolution.

DISCUSSION

Since chilling injury is not caused by physical disruption as in freezing, a number of causes have been postulated; namely, solidification of protoplasmic lipid materials; changes in protoplasmic viscosity, in chemical constitution, in permeability, and in velocities of related metabolic reactions and the accumulation of a toxic compound. Results of the present investigation suggest that changes in the relative amounts of certain metabolic intermediates during the chilling exposure may be involved in chilling injury.

The increased rate of CO_2 evolution observed for orange fruits at 20° C, following exposures to temperatures of 10° and 0° C, is in agreement with reports and reviews on other plant materials mentioned above. Blackman and Parija (5) in 1928 suggested three possible sources of this CO_2: A. "Physical processes; alternation of equilibrium of solution, adsorption and loose chemical union of CO_2"; B. "Carbohydrate equilibrium effect" (considered primarily starch-sugar equilibrium relations; and C. "Intermediate compound effect". Various writers (1, 5, 12, 16, 17) have discussed the relative merits of the first two factors, but few have mentioned the third.

The present study demonstrates a cumulative time-temperature influence of chilling exposure upon the degree of stimulation in CO_2 production at a non-chilling temperature (table Ia). In addition, the total CO_2 content of the fruit is shown to increase, not decrease, after transfer from 0° and 10° C to 20° C (table Ib). The increase in the total CO_2 content is small in comparison with the large amounts of CO_2 evolved by the fruits at 20° C after chilling (tables Ia and b). These results along with the increased

rate of O_2 uptake of immature fruits may be offered as evidence that the CO_2 evolution was of metabolic origin rather than physical desorption of CO_2 associated with the rise of temperature.

The stimulated metabolic activity at 20° C following chilling exposures, as evidenced by the increased rates of CO_2 evolution and O_2 uptake, suggests that reactions leading to oxidation are involved. A possible explanation may be that certain intermediate compounds accumulate in the tissue during the chilling exposure, and when placed at the higher temperature they are available for rapid metabolism. An investigation to evaluate this hypothesis is being initiated. Differential temperature coefficients of some of the various enzymes or enzyme systems could very well account for an accumulation of certain metabolic intermediates at the low temperature. In addition, the accumulation of excessive amounts of certain metabolites may result in physiological upsets and death of the cells, with subsequent development of the typical symptoms of chilling injury. The facts that alteration of holding temperatures between chilling and non-chilling temperatures suppresses chilling injury and that symptoms develop only on the chilled portion of a fruit when half is held at a chilling temperature and the other half is held at a non-chilling temperature (9) are compatible with this hypothesis.

These data and those reported and reviewed elsewhere (1, 8, 16, 17) on the rate of CO_2 evolution at a non-chilling temperature after a holding period at a chilling temperature, generally tend to indicate a difference in the response of plant materials which are susceptible to chilling injury compared with those materials which are not susceptible. Materials sensitive to chilling usually produce a rather large flush of CO_2 at the non-chilling temperature after exposure to chilling temperatures. Materials not sensitive to chilling temperatures usually produce little or no excess CO_2 after exposure to chilling temperatures. This implies a fundamental difference in the physiology and biochemistry of the respiratory mechanism of sensitive and non-sensitive materials.

Summary

The physiological responses of citrus fruits to chilling injury were evaluated by determining the rate of CO_2 evolution, the rate of O_2 uptake, internal atmosphere, and total CO_2 content of chilled and non-chilled citrus fruits at 20° C. A pronounced increase in the rate of CO_2 evolution was associated with the chilling treatments. There was a cumulative time-temperature influence of chilling exposure upon the degree of stimulation in CO_2 evolution. An increase in the rate of O_2 uptake at 20° C of immature orange, lemon, and lime fruits was observed after exposure to 0° and 10° C. Determinations of the composition of the internal atmosphere and total CO_2 content of orange fruits conclusively show that physical processes, such as the release of dissolved CO_2 as the fruits warmed, cannot account for the increased amount of CO_2 involved after a chilling exposure.

It is suggested that the stimulated respiratory rate of chilled fruits may arise from changes in the relative amounts of certain metabolites which are rapidly oxidized at the non-chilling temperature.

Literature Cited

1. Appleman, C. O. and C. L. Smith. 1936. Effect of previous cold storage on the respiration of vegetables at higher temperatures. Jour. Agr. Research 53: 557–580.
2. Biale, J. B. 1942. Preliminary studies on modified air storage of Fuerte avocado fruit. Proc. Amer. Soc. Hort. Sci. 41: 113–118.
3. Biale, J. B. 1950. Postharvest physiology and biochemistry of fruits. Ann. Rev. Plant Physiol. 1: 183–206.
4. Biale, J. B., R. E. Young, and Alice J. Olmstead. 1954. Fruit respiration and ethylene production. Plant Physiol. 29: 168–174.
5. Blackman, F. F. and P. Parija. 1928. Analytic studies in plant respiration. I. The respiration of a population of senescent ripening apples. Proc. Roy. Soc. (London) B. 103: 412–445.
6. Eaks, I. L. 1955. Hydrocooling effect on oranges. Calif. Citrograph 41: 68–70.
7. Eaks, I. L. and W. A. Ludi. Effect of temperature, washing, and waxing on the internal atmosphere of orange fruits. Proc. Amer. Soc. Hort. Sci. Paper No. 967. (In press).
8. Eaks, I. L. and L. L. Morris. 1956. Respiration of cucumber fruits associated with physiological injury at chilling temperatures. Plant Physiol. 33: 308–314.
9. Eaks, I. L. and L. L. Morris. 1957. Deterioration of cucumbers at chilling and non-chilling temperatures. Proc. Amer. Soc. Hort. Sci. 69: 388–399.
10. Eaves, C. A. 1935 Preliminary study of the effect of a series of temperature changes upon respiratory activities of apples during post-climacteric in senescent decline. Sci. Agr. 16: 28–39.
11. Lewis, D. A. 1956. Protoplasmic streaming in plants sensitive and insensitive to chilling temperature. Science 124: 75–76.
12. Lewis, D. A. and L. L. Morris. 1956. Effects of chilling storage on respiration and deterioration of several sweet potato varieties. Proc. Amer. Soc. Hort. Sci. 68: 421–428.
13. Lieberman, M., C. C. Craft, W. V. Audia, and M. S. Wilcox. 1958. Biochemical studies of chilling injury in sweet potatoes. Plant Physiol. 33: 307–311.
14. Miller, E. V. 1946. The physiology of citrus fruits in storage. Bot. Rev. 12: 393–423.
15. Miller, E. V. 1958. The physiology of citrus fruits in storage. II. Bot. Rev. 24: 43–59.
16. Pentzer, W. T. and P. H. Heinze. 1954. Postharvest physiology of fruits and vegetables. Ann. Rev. Plant Physiol. 5: 205–224.
17. Singh, B. N. and P. B. Mathur. 1939. Studies in fruit storage. I. Influence of the stage of maturity and storage temperature on respiratory drifts during the ripening of tomato fruits. Ann. Appl. Biol. 26: 203–212.
18. Willaman, J. J. and W. R. Brown. 1930. Carbon dioxide dissolved in plant sap and its effect on respiration measurements. Plant Physiol. 5: 535–549.

RESPIRATORY RESPONSES TO CATIONS IN A RED ALGA AND THEIR RELATIONSHIPS TO ION TRANSPORT[1, 2]

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Curiosity about high ratios of ions transported to molecules of oxygen consumed in salt respiration in *Porphyra perforata* J. G. Agardh, an intertidal red alga of the Pacific Coast, originally prompted this investigation. It was earlier found that potassium chloride stimulates the respiration of Porphyra tissues washed previously in K-free sea water. The respiratory increase differs, however, from salt respiration in terrestrial plants in its transitory nature and in its specificity for potassium or rubidium. Sodium and lithium give no increase in respiration under these conditions.

Bergquist (2) has shown a similar potassium-induced stimulation of respiration in the brown alga *Homosira banksii* Turn. Decsne. The responses of both algae are similar in time course, but it is not necessary to pretreat Homosira in K-free sea water to obtain increased oxygen consumption on adding potassium.

Examination of these respiratory effects calls forth a number of interesting questions. What is the nature of the K-induced respiratory stimulation and is it related to cation transport? Why is the increase transitory? How can the specificity be accounted for? What relationship does the response bear to salt respiration as it occurs in higher plants?

In seeking answers to these questions a gross requirement for monovalent cations for optimal respiration was found. Absence of K and Na from artificial sea water markedly reduces respiration and subsequent addition of high concentrations of NaCl and KCl restores the initial rate.

Respiratory responses of whole cells to cations, specifically, have been reported earlier for higher plants (10, 11) and for algae (2). The stimulation of respiration on addition of salt, reported in this paper can only be due to cations because in all cases anion concentrations of the artificial sea waters were kept nearly constant and at levels similar to those in natural sea water.

METHODS

Porphyra perforata thalli were collected from rocks of the intertidal zone approximately two miles north of the Los Angeles-Ventura Counties boundary on the California Coast. The plants were kept in plastic bags in a refrigerator up to two weeks before use.

Oxygen consumption rates were determined by conventional Warburg monometry in the dark at 20° C. Twenty 2 cm discs (about 0.5 g) of tissue were placed in each flask. For studies on cyanide inhibition, appropriate concentrations of NaCN were placed in the center wells of the flasks according to Robbie (24).

Methods for determining tissue ion contents and formulae for the artificial sea waters employed are given in a previous paper (9).

RESULTS

IDENTITY OF K EFFECT: A typical respiratory stimulation to added KCl is shown in figure 1. The Porphyra tissues had been soaked 24 hours in K-free sea water before use. The maximum stimulation of respiration in such experiments is about 40 per cent above that in K-free sea water. Half-time for maximum respiration approximates 15 minutes and the duration of stimulation is from 1.5 to 2 hours.

Rubidium chloride (0.02 M) produces an effect which is similar in time course and magnitude to KCl (table I). The effect of cesium chloride (0.02 M)

TABLE I

RESPIRATORY RESPONSES OF PORPHYRA PERFORATA TISSUES, IN K-FREE SEA WATER 24 HOURS, TO ADDED SALTS*

SALT	RESPIRATORY RATES μl O_2/g/20 MIN **		
	BEFORE ADDITION	AFTER ADDITION	INCREMENT DUE TO ADDED SALT
KCl	40	50	10
	43	64	21
	35	51	16
	37	46	9
RbCl	35	47	12
	27	37	10
CsCl	38	43	5
	35	44	9
LiCl	33	32	−1
	34	35	1
K-Free sea water	40	38	−2

* All salts added to 0.02 M.

** Each value represents an average of three readings.

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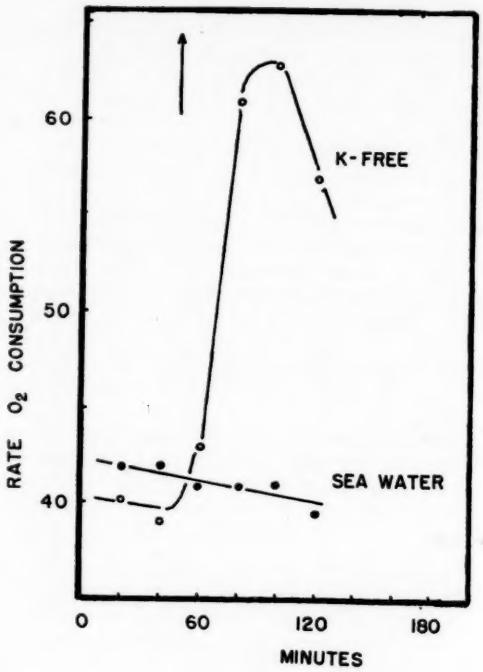
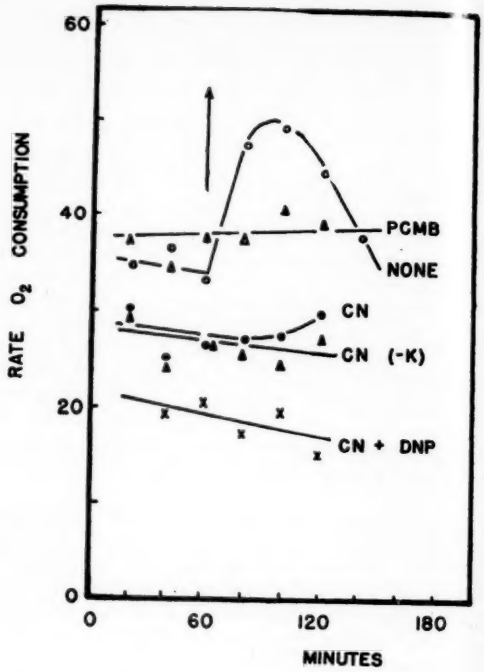
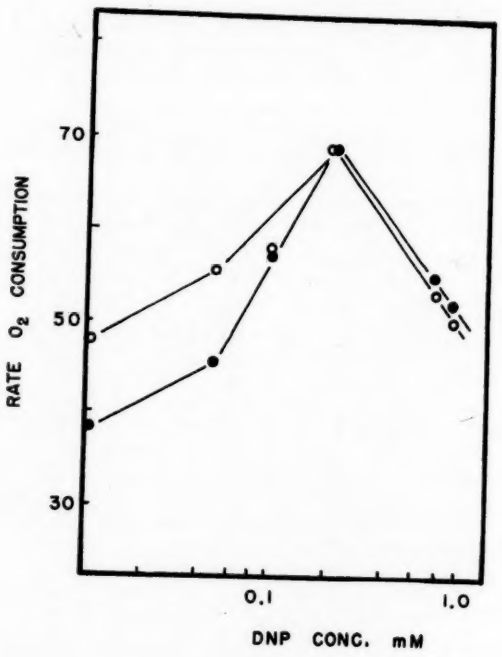

RATE O_2 CONSUMPTION
60
50
40
K-FREE
SEA WATER
0
60
120
180
MINUTES


RATE O_2 CONSUMPTION
60
40
20
0
PCMB
NONE
CN
CN (-K)
CN + DNP
0
60
120
180
MINUTES


RATE O_2 CONSUMPTION
70
50
30
0.1
1.0
DNP CONC. mM

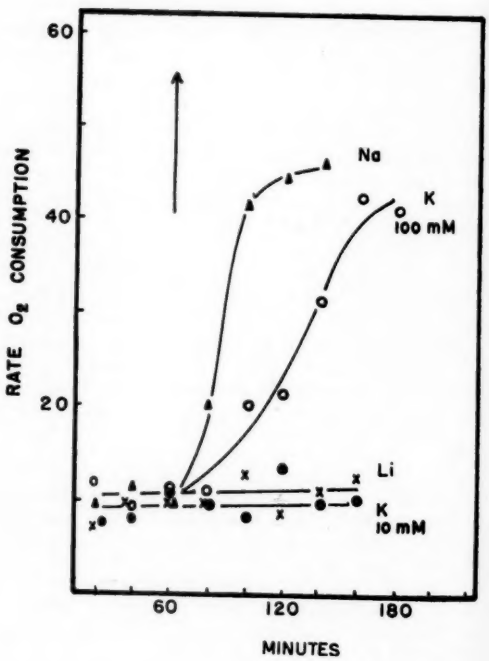

RATE O_2 CONSUMPTION
60
40
20
Na
K
100 mM
Li
K
10 mM
60
120
180
MINUTES

TABLE II

VARIATION IN MAGNITUDE OF RESPIRATORY RESPONSE OF PORPHYRA PERFORATA TISSUES TO ADDED KCL WITH DURATION OF PRETREATMENT IN K-FREE SEA WATER AND K UPTAKE AND NA EXTRUSION IN EXPERIMENTAL PERIOD

HRS IN K-FREE SEA WATER	RESPONSE TO ADDED KCL *	K CONTENTS **		K UPTAKE **	NA CONTENTS **		NA EXTRUSION **
		INITIAL	FINAL		INITIAL	FINAL	
0***	−2	148	183	35	89	93	−4
2.7	6	165	185	20	117	109	8
24	19	87	141	54	136	119	17
48	21	34	102	68	118	90	28

* Difference between averages of three readings before and after adding 0.01 M KCl. Units μL O_2/g fresh wt tissue/20 minutes.

** Units: milli-equivalents/Kg fresh wt of tissue.

*** Samples removed from refrigeration 30 minutes before use; other samples had been some hours at room temperature of 20° C.

is about half that of KCl or RbCl. No effect is produced by LiCl or added K-free sea water (0.5 M in Na).

Soaking tissues in K-free sea water results in loss of K with half-time about 20 hours (8). The magnitude of the respiratory effect of KCl is not proportional to internal K level, however. As little as 2 hours exposure to K-free sea water is sufficient to obtain a measurable response to added KCl (table II).

Curiously, 0.01 M KCl does not cause a respiratory stimulation in the absence of sodium (fig 4). Omission of calcium from the artificial sea water has no influence on the K-effect (data not shown).

EFFECT OF K IN PRESENCE OF INHIBITORS: Sodium cyanide 0.1 mM abolishes the K-effect while reducing respiration in K-free sea water only about 20 per cent (fig 2). *P*-chloromercuribenzoate (PCMB) at 0.5 mM also completely abolishes K-stimulation without inhibiting respiration in K-free sea water at all (fig 2).

2,4-dinitrophenol (fig 3) stimulates respiration in the absence of K, as observed earlier (7). Adding K in the presence of DNP results in further increase in respiration at low DNP concentrations, but gives no additional effect at 0.2 mM DNP, the concentration which gives a maximum respiratory rate, or at higher DNP concentrations. Cyanide abolishes the DNP stimulated respiration as well as the K-effect (fig 2).

K EFFECT AND CATION TRANSPORT: In general, amounts of net K accumulation and Na extrusion increase with increasing magnitude of the respiratory increase to added KCl (table II). However, K accumulation continues after respiration has ceased to be stimulated by added KCl. Cyanide, DNP at concentrations of 0.2 mM, and above, and PCMB all reduce net K and Na transport to varying degrees (table III).

Net extrusion of Na does not occur in the absence of K(8) and the time course of K-stimulation of respiration corresponds roughly to that of net Na extrusion.

To a degree, K accumulation can occur in the absence of a respiratory increase. After storage at 3° C, tissues kept in sea water (with K) take up K on returning them to 20° C without significant respiratory change (table II, see footnote). K accumulation may occur, often at somewhat reduced rates, in Na-free sea water (9) without respiratory stimulation on adding KCl.

RESPIRATORY EFFECT OF NA: Porphyra tissues can be made relatively free of Na- and K-free by soaking overnite in K-free sea water, then in K, Na-free sea water (Mg sea water) for 3 to 4 hours (9). Such tissues show greatly reduced respiration, but the typical rate can be permanently restored by adding 0.1 M NaCl or 0.1 M KCl, but not 0.1 M LiCl or 0.01 M KCl (fig 4).

FIG. 1 (*upper left*). Respiration of *Porphyra perforata* tissues in sea water and K-free sea water. KCl added to 0.01 M at arrow.

FIG. 2 (*upper right*). Respiration of *Porphyra perforata* tissues in K-free sea water with inhibitors. KCl added to 0.01 M at arrow. NaCN 0.1 mM, *p*-chloromercuribenzoate 0.5 mM, 2,4-dinitrophenol 0.2 mM. (-K) indicates no KCl added.

FIG. 3 (*lower left*). Respiration of *Porphyra perforata* tissues in K-free sea water at different DNP concentrations. Points represent average rates for 60 minutes before (solid circles) and after (open circles) adding KCl to 0.01 M.

FIG. 4 (*lower right*). Respiration of *Porphyra perforata* tissues in magnesium sea water (free of K and Na). Salts added at arrow: NaCl 0.1 M (triangles), KCl 0.1 M (open circles), KCl 0.01 M (solid circles), or LiCl 0.1 M (×—×).

TABLE III

EFFECTS OF INHIBITORS ON K AND NA MOVEMENTS, TOTAL AND K-INDUCED RESPIRATION, AND ON ION/O_2 RATIOS IN PORPHYRA PERFORATA

INHIBITOR	CONC. mM	ION MOVEMENTS meq/Kg fresh wt		RESPIRATION mlO_2/Kg fresh wt		ION/O_2 meq/mM			
		K UPTAKE	NA EXTRUSION	TOTAL	K-INDUCED	$K/\Sigma O_2$	$Na/\Sigma O_2$	$K/\Delta O_2$	$Na/\Delta O_2$
None		49*	33	237	61	4.8	3.3	19.5	13.9
		24–74**	22–57	211–264	23–84	2.7–6.7	1.4–6.4	13–28	4.5–24
Cyanide	0.1	31	18	122	...	6.6	3.5		
		12–39	9–25	91–136	...	2.2–10	2.3–4.8		
DNP	0.2	17	6	380	...	1.1	0.4		
		9–23	3–10	377–388	...	0.6–1.4	0.2–0.6		
	1.0	11	−4***	272	...	1.0	−0.3		
		6–17	−11–0	262–290	...	0.5–1.6	−1.0–0.2		
PCMB	0.5	14	15	143	...	1.3	1.4		
	1.0	...	5	169	...	...	0.7		

* Average.
** Range. Number of samples: No inhibitor, 14; Cyanide, 4; DNP, 3 at each concentration; PCMB, 1 only.
*** Minus sign indicates Na gain in samples.

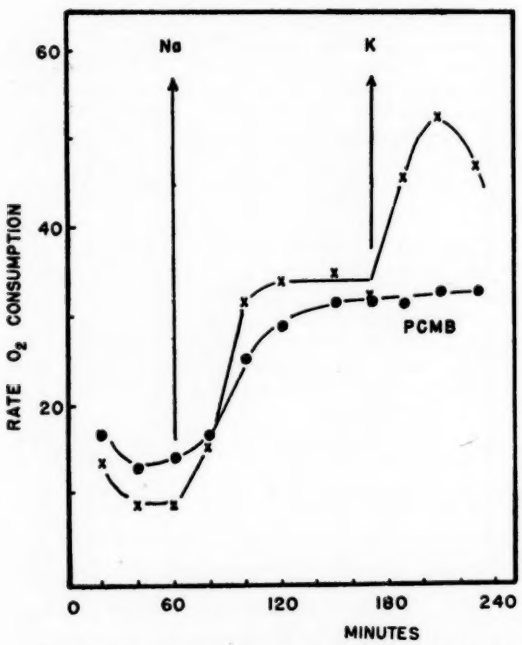


FIG. 5 (*above*). Respiration of *Porphyra perforata* tissues in magnesium sea water (free of K and Na) with or without PCMB 0.5 mM. NaCl added at first arrow to 0.1 M, KCl added at second arrow to 0.01 M.

That the effect of added NaCl is distinct from the K-effect previously described is shown in figure 5. The respiratory rate is low in K, Na-free sea water and is restored to the typical rate on addition of NaCl. Subsequent addition of 0.01 M KCl results in further increase in rate with the usual transient time course.

Lithium chloride will not produce the effect. Unlike Na, Li is not extruded by Porphyra (9), although it may be by frog skin (30) for example.

INFLUENCE OF INHIBITORS ON NA EFFECT: PCMB, although completely effective in abolishing the K-effect, scarcely influences the respiratory recovery allowed by added NaCl (fig 5). Cyanide reduces the maximum effect of NaCl, but the final rate approximates that in K-free sea water with cyanide (fig 6). Cyanide also greatly slows the recovery in respiratory rate on adding NaCl, however.

In the presence of DNP, at optimum concentration, adding Na results in further increase in respiration rate above that induced by DNP (fig 6).

INFLUENCE OF OTHER CATIONS ON RESPIRATION: Tissues soaked in Ca-free sea water or Mg-free sea water show typical respiratory rates (105–120 μl O_2/g fresh wt/hr) which are not altered by adding $CaCl_2$ or $MgCl_2$, respectively. Either these cations are without influence on rate limiting reactions or the pretreatments used did not remove Ca or Mg from critical sites. These possibilities were not investigated.

Discussion

Respiratory Effect of Potassium: The bulk of respiration of Porphyra in K-free sea water is insensitive to 0.1 mM cyanide suggesting that electron transport by cytochrome oxidase is not rate limiting under these conditions. Rather, because of the stimulation of respiration by DNP, oxidative phosphorylation appears to be rate limiting. However, when KCl is added to tissues in K-free sea water the resulting respiratory stimulation is cyanide sensitive. This suggests that oxidative phosphorylation is no longer limiting the rate of oxygen consumption. These findings suggest that K stimulates respiration via oxidative phosphorylation rather than cytochrome oxidase.

Other explanations for the action of K have also been considered. There has been a report of an uncoupling effect of K, at certain concentrations, in muscle (3) which could allow these results to obtain. This seems unlikely in Porphyra because at least one energy-requiring process, sodium extrusion, occurs at maximal rates on addition of K to tissues in K-free sea water (8).

Another possible explanation might be that K allows the operation of a new respiratory pathway. Laties (15, 16) has described the appearance of a new type of respiration on ageing of chicory root slices. The new respiration is malonate sensitive. Malonate and malonic ester were without effect on the K-stimulated respiration in Porphyra. However, PCMB selectively abolishes the K-induced increment, as does cyanide.

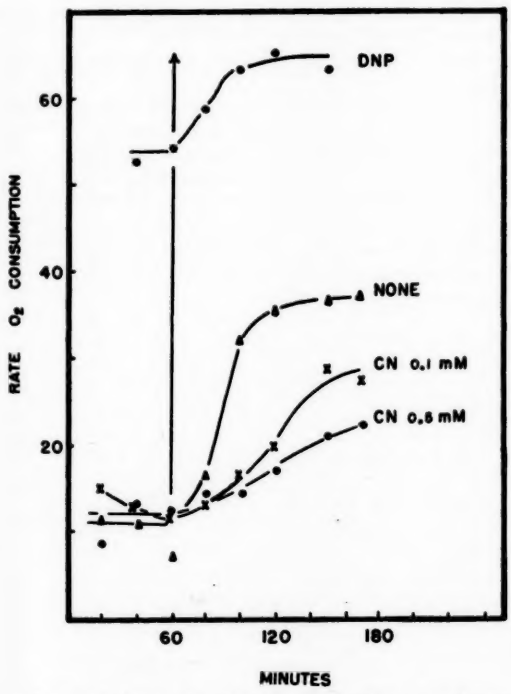


Fig. 6 (*below*). Respiration of *Porphyra perforata* tissues in magnesium sea water (free of K and Na) with NaCN, no inhibitor, or DNP 0.2 mM. NaCl added at arrow to 0.1 M.

The resemblance of K-stimulated respiration in Porphyra to the respiratory rise on ageing storage tissues seems only superficial when time courses are considered. The rise in storage tissues is permanent and requires several hours for maximum development while K-stimulated respiration in Porphyra is transient and reaches a maximum value only 20 to 40 minutes after adding KCl. These results make the supposition of a special K-stimulated respiratory pathway unattractive.

Comparison of the K-stimulated respiration in Porphyra with the effects of K on respiration of isolated mitochondria allow an explanation based on K-stimulation of oxidative phosphorylation to appear most reasonable. Pressman and Lardy (21) found that K notably stimulates respiration of rat liver mitochondria in the presence of phosphate acceptors. The response to K was not additive to the stimulation of respiration obtained at optimal DNP concentration. Pressman and Lardy concluded that K probably acts by influencing phosphate transfers and not upon oxidative phosphorylation directly. Lithium and sodium did not give the K-induced stimulation but rubidium gave a response similar to K (20). The same specificity is seen in Porphyra.

An hypothesis based upon K activation of phosphate transfers allowing increased respiration of Porphyra cells seems justified in the light of the above findings. The transient nature of the response must represent, in this interpretation, a rapid increase followed by a gradual decline of available phosphate acceptors on adding KCl. In the absence of K ATP or similar high-energy phosphate compound would not be utilized at maximal rates. Adding K would increase ATP utilization resulting in increased P-acceptors only until a new steady-state concentration was established, or until any metabolic work utilizing high-energy phosphate was completed.

An example of a similar transitory change in respiration is to be seen in Syrett's study of respiratory rate and ATP concentration in Chlorella (28). In this case addition of ammonium ions to N-starved cells stimulated transient respiration. The measured ATP concentration initially fell on adding ammonium salt, then increased to a constant value as the initial respiratory rate was restored.

Also of interest is the finding of cation specific ATP-ase activity in certain crab nerves (27) and in human erythrocyte ghosts (22). Potassium and sodium are both required for optimal activity of these enzymes; activity would result in increased supply of P-acceptors.

Achievement of a transitory respiratory increase on adding KCl to tissues soaked in K-free sea water less than 4 hours (while internal K is at least 80 % as great as control tissues) suggests that K initially leaves critical sites, which might be located near the

cell surface. If this is true, then it is likely that the reactions leading to production of P-acceptors are also near the cell surface (presuming that K stimulates the reactions involved).

There is considerable evidence that high-energy phosphate compounds are involved in ion transport. ATP injected into squid giant axons stimulates extrusion of Na (4). Both K and Na activated ATPase of red blood cell ghosts and K and Na transport of the intact cells are inhibited by ouabain and require the presence of both K and Na (22). Results such as these suggest that utilization of ATP at the cell surface is quite possible, as is the generation of P-acceptors in the process of performing ion transport. It is thus feasible to speculate that net extrusion of Na by Porphyra cells, which occurs only in the presence of K(8), produces P-acceptors which allow an increased rate of oxidative phosphorylation resulting in increased oxygen consumption. Evidence supporting this notion comes from the similarity between the time course of Na extrusion and that of K-stimulated respiration. It is obvious that K may stimulate a number of ATP requiring systems other than Na extrusion, however.

Respiratory Effect of Sodium: The respiratory increase of Porphyra soaked in Mg-sea water (free of K and Na) on adding 0.1 M NaCl or KCl is different from that obtained on adding 0.01 M KCl to tissues soaked in K-free sea water. Adding Na to K, Na-free tissues results in increased respiration even in the presence of optimal concentrations of DNP. The effect of Na is only partially inhibited by cyanide and is little influenced by PCMB. It is not transitory but permanent. Further, absence of cell K and Na much reduces respiration while absence of K alone does not. These results suggest direct stimulation of respiration by Na and high concentrations of K.

Lehninger and Kennedy (18) found that rat liver mitochondria require the presence of a neutral salt (apparently with monovalent cation) for optimum respiration. Either KCl or NaCl would serve to increase oxygen consumption. This effect probably represents a gross requirement for monovalent cations and is quite distinct from the K-specific stimulation of respiration in the presence of P-acceptors.

The results with mitochondria seem analogous to the requirement of high concentrations of Na or K for maximum steady-state respiration in Porphyra. Curiously lithium will not do. In both mitochondria and Porphyra cells absence of monovalent cation results in greatly reduced oxygen consumption and addition of salt restores the respiration rate.

Because DNP stimulates respiration in the absence of monovalent cations, a part of the effect of high K or Na may represent stimulation of oxidative phosphorylation. But because adding Na in the presence of DNP further increases respiration, part of the high salt effect may involve stimulation of electron transport.

It seems reasonable that cells approximately 0.5 M in monovalent cation (mostly K) would show decreased enzymatic activity in its absence. Thus direct effects of salt seem to be reasonably expected and probably account for the gross salt requirement in this case.

Salt Respiration in Porphyra Compared With Higher Plants: The K-induced respiratory stimulation in Porphyra previously kept in K-free sea water is clearly distinct from salt respiration in higher plants in its specificity for K, Rb, or to a lesser extent Cs, and in its transient nature. In both higher plants and Porphyra the salt respiration is cyanide sensitive (25) and does not appear in the presence of optimal concentrations of DNP (26).

The Na, or gross salt, effect in Porphyra is also somewhat unique for whole cells. The most striking feature is that the respiratory rate is so greatly depressed in the absence of monovalent cations and is restored so quickly in the presence of 0.1 M NaCl or KCl. Cyanide is less effective in abolishing the Na effect in Porphyra than in blocking salt respiration in carrot discs (25, 29).

There are, however, two points of similarity in the Na effect in Porphyra and typical salt respiration. The Na effect in Porphyra is to a degree additive to DNP stimulation, and Honda (13) indicates independence of DNP and salt stimulated respiration in roots. Handley and Overstreet (11) provide evidence that salts stimulate respiration of barley roots directly, which is also the case for the Na effect in Porphyra.

The K effect and the gross salt effect in Porphyra and salt respiration in higher plants (14) share in the likelihood that oxidative phosphorylation is a site of action. The differences seem to lie in how this is brought about, i.e., directly or indirectly by increasing the supply of P-acceptors. In addition, the gross salt requirement in Porphyra may represent a composite effect upon oxidative phosphorylation and electron transport.

Salt Respiration and Ion Transport in Porphyra: The Na effect is clearly independent of active K or Na transport because the presence of K is necessary for both K accumulation and Na extrusion (8).

It also seems unlikely that net K accumulation has any relationship to the K effect. K accumulation may proceed after cessation of K-stimulated respiration and may also occur on warming chilled tissues in the absence of any respiratory increase. (Some time must elapse before the respiratory rate of chilled tissues returns to normal on warming, but K may be accumulated during this warming period. Na, however is not extruded).

The possibility remains, as mentioned earlier, of a relationship between Na extrusion and the K effect. The time courses of the processes are similar; and there is no effect of K when Na extrusion is prevented as in the presence of cyanide, PCMB, or when Na is at a very low level in the cells or the medium.

A striking feature of the K effect and of transport is the specificity manifested toward cations. It is tempting to speculate that the respiratory systems which display this specificity are those which confer specificity upon cation transport! The author finds it more satisfying to imagine that transport specificity is determined by ion-specific enzymatic processes, such as the K, Na influenced ATP-ases cited earlier, than to think in terms of specific ion-binding carriers of probably low molecular weight.

RATIOS OF CATIONS TRANSPORTED TO MOLECULES OF OXYGEN CONSUMED IN RESPIRATION OF PORPHYRA: Lundegardh's (19) and Conway's (5) redox theories of ion transport demand that no more than four ions be transported actively per oxygen molecule consumed in respiration. Ratios greater than four have been reported for both plant and animal tissues (11, 17, 6, 31).

In Porphyra ratios greater than four are obtained when K and Na transport are equated to the K-stimulated respiratory increment (table III). However, when transported ions are equated to total respiration only the K/O_2 ratio exceeds four, on the average. The value of these data may be challenged on a number of bases: A. Passive movements have not been determined concurrently; B. Only one of the ions, K or Na, must be actively transported, although both may be; C. The errors involved in obtaining the ratios are large. The data then suggest that a redox theory may be valid only if total respiration, and not the K-induced increment, is considered.

A redox theory can be considered valid if it is assumed that K, acting as a cofactor for transport, allows most of the available respiratory energy to be utilized for net transport, at least for short periods. There is considerable evidence that K may act as a cofactor for transport. Squid giant axons (12) and Porphyra cells require K for Na extrusion. Amino acid transport in tumor cells (23) and sugar transport in some bacteria (1) also require K. Unfortunately no data are available concerning what portion of respiratory energy may be utilized by Porphyra for ion transport.

The evidence at hand is equally good, if not better in support of a theory of transport involving utilization of high-energy phosphate compounds. Low ratios of ions transported to molecules of oxygen consumed in the presence of DNP and PCMB as compared with cyanide favor such a view (table III). The possibility of cation specific ATP-ase activity conferring specificity upon, and resulting in, transport is also attractive at the moment.

SUMMARY

I. Potassium, rubidium, and to a lesser extent cesium chlorides stimulate respiration of the red alga *Porphyra perforata* previously soaked in K-free sea water. The stimulation is transient, reaching a maximum 20 to 40 minutes after adding the salt and falling rapidly thereafter. The respiratory effect of KCl is abolished by cyanide and *p*-chloromercuribenzoate; adding KCl gives no further increase at levels of 2,4-dinitrophenol which induce maximum respiration. Possible mechanisms of KCl stimulation are discussed; it is concluded that K probably acts by stimulating phosphate transferring reactions.

II. Respiration of Porphyra is greatly reduced in magnesium sea water lacking monovalent cations. Typical respiratory rates are restored by adding NaCl or KCl, but not LiCl, to 0.1 M. This effect probably represents a direct requirement of the respiratory system for monovalent cations for optimal activity.

III. Both types of salt respiration are independent of K accumulation, but the possibility remains that the K-specific stimulation represents an indirect effect via stimulating Na extrusion.

IV. Ratios of net K accumulation and Na extrusion to oxygen consumption in K-stimulated respiration are variable, and greater than four. The possibility that adding K may allow a larger fraction of respiratory energy to be available for net Na extrusion must also be considered because of the possible role of K as a cofactor for Na extrusion.

LITERATURE CITED

1. ABRAMS, A. and C. G. MACKENZIE. 1958. The role of potassium and sodium and glycolysis in the transport mechanisms of bacterial cells and protoplasts. Fed. Proc. 18: 178.
2. BERGQUIST, P. L. 1959. The effect of cations and anions on the respiratory rate of the brown alga, *Homosira banksii*. Physiol. Plantarum 12: 30–36.
3. BRUNER, G. P., S. E. SIMON, R. FRATER, and P. TASKER. 1959. A comparison of ion shifts with adenosine triphosphate and creatine phosphate levels in muscle. Biochim. Biophys. Acta 35: 485–495.
4. CALDWELL, P. C. and R. D. KEYNES. 1957. The utilization of phosphate bond energy for sodium extrusion from giant axons. Jour. Physiol. 137: 12P.
5. CONWAY, E. J. 1953. A redox pump for the biological performance of osmotic work, and its relation to the kinetics of free ion diffusion across a membrane. Internat. Rev. Cytol. No. II: 419–445.
6. DAVIES, R. E. 1954. Relations between active transport and metabolism in some isolated tissues and mitochondria. Symp. Soc. Exp. Biol. No. VIII: 453–475.
7. EPPLEY, R. W. 1958. Sodium exclusion and potassium retention by the red marine alga, *Porphyra perforata*. Jour. Gen. Physiol. 41: 901–911.
8. EPPLEY, R. W. 1958. Potassium-dependent sodium extrusion by cells of *Porphyra perforata*, a red marine alga. Jour. Gen. Physiol. 42: 281–288.
9. EPPLEY, R. W. 1959. Potassium accumulation and sodium efflux by *Porphyra perforata* tissues in lithium and magnesium sea waters. Jour. Gen. Physiol. 43: 29–38.
10. EPSTEIN, E. 1954. Cation induced respiration in barley roots. Science 120: 987–988.
11. HANDLEY, R. and R. OVERSTREET. 1955. Respiration and salt absorption by excised barley roots. Plant Physiol. 30: 418–426.

12. HODGKIN, A. L. and R. D. KEYNES. 1955. Active transport of cations in giant axons from Sepia and Loligo. Jour. Physiol. 128: 28–60.
13. HONDA, S. I. 1956. The salt respiration and phosphate contents of barley roots. Plant Physiol. 31: 62–70.
14. LATIES, G. G. 1957. Respiration and cellular work and the regulation of the respiratory rate in plants. Survey of Biol. Progress No. 3: 215–299.
15. LATIES, G. G. 1959. The nature of the respiratory rise in slices of chicory root. Archiv. Biochem. Biophys. 79: 364–377.
16. LATIES, G. G. 1959. The development and control of coexisting respiratory systems in slices of chicory root. Archiv. Biochem. Biophys. 79: 378–391.
17. LEAF, A. and A. RENSHAW. 1957. Ion transport and respiration of isolated frog skin. Biochem. Jour. 65: 82–90.
18. LEHNINGER, A. L. and E. P. KENNEDY, 1948. The requirements of the fatty acid oxidase complex of rat liver. Jour. Biol. Chem. 173: 753–777.
19. LUNDEGARDH, H. 1955. Mechanisms of absorption, transport, accumulation, and secretion of ions. Ann. Rev. Plant Physiol. 6: 1–24.
20. PRESSMAN, B. C. and H. A. LARDY. 1952. Influence of potassium and other alkali cations on respiration of mitochondria. Jour. Biol. Chem. 197: 547–556.
21. PRESSMAN, B. C. and H. A. LARDY. 1955. Further studies on the potassium requirements of mitochondria. Biochim. Biophys. Acta 18: 482–487.
22. POST, R. L. 1959. Relationship of an ATP-ase in human erythrocytes to the active transport of sodium and potassium. Fed. Proc. 18: 121.
23. RIGGS, T. R., L. M. WALKER, and H. N. CHRISTENSEN. 1958. Potassium migration and amino acid transport. Jour. Biol. Chem. 233: 1479–1487.
24. ROBBIE, W. A. 1948. Use of cyanide in tissue respiration studies. In: Methods in Medical Research. Vol. 1. Pp. 307–316. Year Book Publishers, Chicago.
25. ROBERTSON, R. N. and J. S. TURNER. 1945. Studies on the metabolism of plant cells. 3. The effects of cyanide on the accumulation of potassium chloride and on respiration; the nature of the salt respiration. Australian Jour. Exp. Biol. Med. Sci. 23: 63–73.
26. ROBERTSON, R. N., M. J. WILKINS, and D. C. WEEKS. 1951. Studies on the metabolism of plant cells. 9. The effects of 2,4-dinitrophenol on salt accumulation and salt respiration. Australian Jour. Sci. Res. B. 4: 248–264.
27. SKOU, J. C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. Biophys. Acta 23: 394–401.
28. SYRETT, P. J. 1958. Respiration rate and internal adenosine triphosphate concentration in Chlorella. Archiv. Biochem. Biophys. 75: 117–124.
29. TURNER, J. S. and V. F. HANLY. 1949. Succinate and plant respiration. New Phytol. 48: 149–171.
30. ZERAHN, K. 1955. Active transport of lithium in isolated frog skin. Acta Physiol. Scand. 33: 347–358.
31. ZERAHN, K. 1956. Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. Acta Physiol. Scand. 36: 300–318.

ANTHOCYANIN SYNTHESIS IN CORN ENDOSPERM TISSUE CULTURES. II. EFFECT OF CERTAIN INHIBITORY AND STIMULATORY AGENTS[1, 2]

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In a previous paper (7), the advantages of corn endosperm tissue cultures as an experimental tool in attacking the problem of anthocyanin synthesis were pointed out. The general culture conditions, the identity of the pigments, and the requirement for light were reported in the same paper.

Thimann and Radner (11, 12) have shown that anthocyanin synthesis in Spirodela is inhibited by purine and pyrimidine antagonists, by certain sulfur-containing compounds and certain amino acids. They also demonstrated (13) that riboflavine reverses the inhibition caused by many of these substances, and further that riboflavine substitutes for the light reaction involved in anthocyanin production by Spirodela.

When work on the current problem was begun, it was expected that the effect of various chemicals on anthocyanin synthesis in both the endosperm cultures and Spirodela would prove to be similar. As this report will indicate, however, data obtained so far show that pigment synthesis in the two systems is affected differently by certain of the same compounds tested.

MATERIALS AND METHODS

Tissue cultures derived from endosperm of the corn variety, Black Mexican Sweet, were used in this study. The method of preparing the explants, general culture conditions, and determinations of weight and pigment concentration have been described previously (7). The culture medium used in the present study has been modified from that used in the previous investigation. Tissues were analyzed 25 days after transfer to the experimental media.

Originally the medium contained 0.5 % Seitz-filtered yeast extract. In some of the experiments described here, the yeast extract was omitted and in its stead 0.2 % casein hydrolyzate (acid-hydrolyzed, vitamin-free) adjusted to pH 6.6 with KOH was substituted. In other experiments, asparagine at a concentration of 1.5×10^{-2} M was substituted for the yeast extract.

At the same time the experiments on pigment synthesis were undertaken, intensive work was being done to develop a completely synthetic medium for the tissue. It was found that asparagine at a concentration of 1.5×10^{-2} M added to the basal mineral-sugar-vitamin medium supported better growth than either yeast extract or casein hydrolyzate. Details of the development of this synthetic medium are being published elsewhere (8).

Although asparagine supports better growth than either of the complexes used, pigment production is depressed on media containing it. Thus in those experiments in which casein hydrolyzate was used as the organic nitrogen source, the controls are casein hydrolyzate media; in those experiments in which asparagine was substituted for casein hydrolyzate, the controls are asparagine media. Experiments are now in progress to repeat certain of the inhibition studies with asparagine media to determine if there is in fact any qualitative difference in response to inhibiting agents by the tissue on casein hydrolyzate and asparagine media.

Riboflavine was determined by the methods described by Strong (9) utilizing the modifications of Thimann and Radner (13) for determining free and bound riboflavine. Difco riboflavine assay medium was used throughout. Growth of the bacteria was determined turbidimetrically by means of a Klett colorimeter employing a red filter (transmitting at 640–700 mμ).

RESULTS

EFFECT OF NUCLEOTIDES: In one attempt to provide a synthetic medium for growth of the tissue, various combinations of the known constituents of yeast extract were tested. Among these was a mixture of adenylic, cytidylic, guanylic, and uridylic acids. All were tested at a concentration of 50 mg/l. The pH of the medium was adjusted to 6.6 with KOH. These nucleotides had very little effect on growth, but caused a considerable increase in pigment production (table I). Part of the increase in pigment may be attributed to the arrested growth and consequent accumulation of anthocyanin by the tissues. When the amount of pigment produced on this medium is compared with that produced by tissue deprived of organic nitrogen, however, there is in fact an increase in pigment which may be attributed to the nucleotides.

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[2] Supported by a grant (G-3529) from the National Science Foundation.

TABLE I

EFFECT OF NUCLEOTIDES ON PIGMENT PRODUCTION

NUCLEOTIDE*	% INCREASE FRESH WT	% OF CONTROL; PIGMENT**
None (control)	53	100
Adenylic acid	60	145
Cytidylic acid	58	122
Uridylic acid	50	106
Guanylic acid	58	100
Mixture of all four nucleotides	45	152

* All nucleotides were added at 50 mg/l.
** Control medium in this case consists of the basal medium without organic nitrogen.

To determine whether or not all four were equally effective, separate media were prepared, each of which contained a single nucleotide at a concentration of 50 mg/l. The results showed guanylic and uridylic acids were without effect. Adenylic acid caused an increase in pigment of 145 % of the controls, while cytidylic acid caused an increase of 122 %.

EFFECT OF PURINE AND PYRIMIDINE ANALOGUES: These results do not indicate a direct participation of the nucleotides in pigment synthesis. The possibility existed that their stimulating action was non-specific. One method to test this possibility would be to attempt to induce inhibitions of pigment production by means of purine and pyrimidine analogues. In addition, the results of Thimann and Radner (12) demonstrated that this class of inhibitors does in fact strongly affect pigment production in Spirodela. Hence, a survey of these substances was made in connection with the endosperm tissues. The data are summarized in table II. All the antimetabolites were added at a concentration of 5×10^{-5} M.

None of the effects on pigment production is very great with these substances at a concentration of 5×10^{-5} M. Higher concentrations up to 5×10^{-4} M are indeed more inhibitory, but are extremely toxic to the tissues. These results were in general rather surprising in view of Thimann and Radner's work (12). They found, for example, that azaguanine was 650 times more effective as an inhibitor than thiouracil. The 50 % inhibition point was at 6×10^{-8} M. At a concentration of 10^{-6} M, pigment formation was inhibited 85 %. There is no question about the uptake of azaguanine by the endosperm tissues since a definite decrease in the amount of growth is manifest. The same may be said of 2,6-diaminopurine, its aza-analogue, thiouracil, azaadenine, and azauracil. The only one of these analogues which shows appreciable pigment inhibition is thiouracil. Hence more detailed studies were made only of thiouracil. Figure 1 shows the effect of varying concentrations of thiouracil on growth and pigment production.

TABLE II

EFFECT OF PURINE AND PYRIMIDINE ANTIMETABOLITES

ANTIMETABOLITE*	% OF CONTROLS**	
	GROWTH	PIGMENT
2,6-Diaminopurine	45	81
Azathymine	106	79
Azaxanthine	95	78
Thiouracil	67	70
Thioadenine	128	98
Azaadenine	43	100
Thiocytosine	121	106
Mercaptopurine	100	100
Azauracil	71	145
Azaguanine	67	112
Aza-2,6-diaminopur.ne	74	132

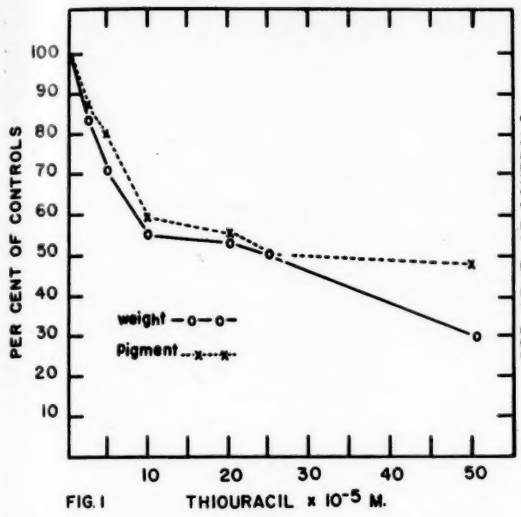
* All at 5×10^{-5} M.
** Controls are casein hydrolyzate medium.

At a concentration of 5×10^{-4} M thiouracil inhibited anthocyanin synthesis by 52 % and growth by 70 %. To reverse these inhibitions, uracil was tested at different concentrations while the thiouracil concentration was kept constant at 5×10^{-4} M. From the data in figure 2, it can be seen that uracil at 10^{-3} M completely reverses the growth inhibition, but increases pigment to only 61 % of the controls. Trials of media containing only uracil at each of the concentrations used in connection with thiouracil showed no effects on either growth or pigment. Above 10^{-3} M to 10^{-2} M, however, uracil begins to inhibit both growth and pigment to some degree. Thus while some reversal of pigment inhibition could be obtained with uracil, the number of useful experiments which could be performed along these lines was very small due to the high concentrations of the substances involved and the appearance of toxic symptoms.

These data taken as a whole indicate that there is little or no direct participation of purines and pyrimidines in the synthesis of anthocyanin in the endosperm tissues. The promotive effects of adenylic and cytidylic acids therefore probably indicate an indirect role.

EFFECT OF RIBOFLAVINE: Thimann and Radner (13) have demonstrated that riboflavine substitutes for the light reaction in the synthesis of anthocyanin in Spirodela. It was shown further that riboflavine causes the reversal of the pigment inhibition caused by many of the inhibitors used in their study. Analyses of the Spirodela plants showed that the riboflavine content was decreased in the presence of many of the inhibitors. In view of these results, various concentrations of riboflavine were tested with the endosperm tissue both in the light and the dark.

The data presented in table III show that riboflavine begins to induce inhibition of pigment production in these tissues at concentrations as low as 10^{-6} M both in the light and the dark. Thimann and Radner reported that concentrations of riboflavine above 10^{-4} M inhibited anthocyanin formation in Spirodela. Thus, it was possible that the endosperm tissues synthesized a large amount of riboflavine and any exo-



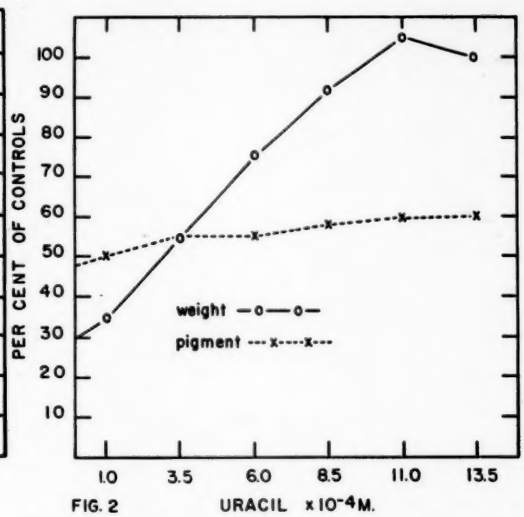


FIG. 1. The effect of varying concentrations of thiouracil on pigment formation and growth. Controls are tissues grown on casein hydrolyzate medium.

FIG. 2. The effect of uracil on reversing the inhibition of growth and pigment formation caused by thiouracil. Thiouracil concentration is constant at 5×10^{-4} M. Controls are tissues grown on casein hydrolyzate medium.

genous supply raised the total riboflavine content to an inhibitory level within the tissues. Consequently, the endosperm tissues were analyzed for their riboflavine content. The amounts of riboflavine found in tissues grown on yeast extract, casein hydrolyzate, and asparagine media were found to vary between 2.03 and 2.10 μg/g fresh weight. Interestingly enough, tissue grown on asparagine media consistently showed the higher concentration of riboflavine. These results were obtained for free riboflavine. Analyses for bound riboflavine were completely negative. Apparently, all of the vitamin is removed from the endosperm tissue by the modified extraction process described by Thimann and Radner. Hence the total riboflavine content of the tissue may be considered to be between 2.03 and 2.10 μg/g. Thimann and Radner reported the Spirodela inoculum to contain a total (free and bound) of 1.39 μg riboflavine per gram fresh weight. There was an increase in total riboflavine content of 0.49 μg at the end of 5 days which is the normal length of their experimental periods. Thus, the total riboflavine content of Spirodela is 1.88 μg/g. Since the endosperm tissues were also analyzed at the end of the normal experimental period used for them of 25 days, the difference in total riboflavine content is only between 0.15 and 0.22 μg/g for the two tissues.

TABLE III

EFFECT OF RIBOFLAVINE ON PIGMENT FORMATION IN LIGHT AND DARK

RIBOFLAVINE CONC MOLAR	PIGMENT, % OF CONTROLS*	
	LIGHT	DARK
10^{-6}	87	90
5×10^{-6}	65	83
10^{-5}	64	76
5×10^{-5}	58	70
10^{-4}	41	42
5×10^{-4}	47	24

* Controls are casein hydrolyzate medium.

It is doubtful that this relatively small difference could account for the opposite reactions of the two tissues in response to exogenous riboflavine unless there is a very narrow optimum range of internal riboflavine concentrations which permits maximum pigment synthesis. This appears unlikely since Thimann and Radner have demonstrated that when Spirodela was floated on solutions of a mixture of azaguanine and riboflavine for 5 days, the total riboflavine content per gram fresh weight was 2.32 μg. In this experiment it was shown that the vitamin could reverse almost fully the inhibition caused by azaguanine.

EFFECTS OF AMINO ACIDS: When asparagine was discovered to be the key to the synthetic medium as described earlier in this report, it was found to support better growth than either casein hydrolyzate or yeast extract. At the same time, anthocyanin formation was considerably less on the asparagine medium. The per cent increase in fresh weight on casein hydrolyzate medium is 230; on yeast extract it is 280, and on asparagine (1.5×10^{-2} M) it is 360. The

number of pigment units per gram of tissue (fresh wt) is, respectively: 1,170, 1,520, and 470. At a concentration of 3.7×10^{-3} M, asparagine permits an increase in fresh weight of 192 % and 590 pigment units per gram of tissue. This represents 84 % of the growth and 50 % of the pigment obtained on casein hydrolyzate medium. Thus, the decrease in pigment production cannot be ascribed entirely to the increase in growth rate and subsequent dilution of the pigment.

Since anthocyanin synthesis on asparagine medium was so low, the possibility arose that perhaps one or more amino acids (or peptides) present in casein hydrolyzate or yeast extract were specifically responsible for the higher pigment concentrations developed on media containing the latter two complexes. Another obvious possibility was that asparagine was specifically inhibitory. To test this, a series of media was prepared, each of which contained a single amino acid at a concentration of 1.5×10^{-2} M. A duplicate series of media was prepared which contained the single amino acid plus asparagine, both at 1.5×10^{-2} M. A total of 21 amino acids including glutamine was tested in this manner. All were used as the 1-form and were autoclaved except for tryptophan which was sterilized by Seitz-filtration. The pH of the media was adjusted to 6.6. In the case of cystine and tyrosine, saturated solutions were used because of their low solubility. The concentrations of these two amino acids based on their known solubilities were calculated to be approximately 2.3×10^{-3} M for tyrosine and 4.6×10^{-4} M for cystine. About half the amino acids had very little effect or were extremely toxic to the tissues. Lysine and tryptophan caused the death of the tissues. Underhill, et al (14) demonstrated that phenylalanine was an excellent precursor in the synthesis of the B-ring of quercetin by buckwheat. This amino acid was tested with the endosperm cultures. It had a slight, possibly insignificant, inhibitory effect on pigment synthesis, but was very inhibitory to the growth of the tissue. The other amino acids which seemed to evoke meaningful responses are listed in table IV.

TABLE IV

EFFECT OF AMINO ACIDS

AMINO ACID*	% OF CONTROLS** AMINO ACID ALONE		% OF CONTROLS** AMINO ACID PLUS ASPARAGINE	
	GROWTH	PIGMENT	GROWTH	PIGMENT
None	16	230	...	...
Aspartic acid	32	750	95	160
Cystine	20	560	71	161
Glutamic acid	30	350	85	101
Proline	62	308	90	110
Cysteine	27	240	86	109
Tyrosine	10	216	100	89
Arginine	21	86	77	63
Serine	21	75	97	79
Valine	22	72	89	51
Glutamine	38	46	96	47
Methionine	17	33	47	30

* All at 1.5×10^{-2} M except for cystine (4.6×10^{-4} M) and tyrosine (2.3×10^{-3} M).
** Controls are asparagine media.

There are several interesting results. Aspartic acid and cystine strongly promote pigment production alone. That this is not an accumulation of pigment by slow growing cultures is obvious when the pigment concentration of cultures maintained on media devoid of organic nitrogen is compared (table IV). In combination with asparagine, there is also an appreciable promotion of anthocyanin synthesis. The mixture of aspartic acid and asparagine may be directly compared with asparagine alone since there is no significant effect on growth caused by the mixture. Cystine in combination with asparagine inhibits growth and hence part of its pigment promotion in this case may be due to an accumulation of pigment by the slower growing tissues. Glutamine has no effect on growth in combination with asparagine, but inhibits pigment formation alone and in combination by over 50 %. Glutamic acid alone promotes pigment synthesis, but in combination with asparagine, it inhibits growth to some degree. Methionine, which was shown by Thimann and Radner (11) to be a strong inhibitor for Spirodela, retards both growth and pigment quite markedly in the endosperm cultures. Arginine, serine, and valine inhibit pigment both alone and in combination with asparagine. Of these three, serine has very little effect on growth: of the remaining two, arginine inhibits growth by 23 % and valine by 11 %. Tyrosine inhibits pigment formation only slightly. This may be due to its lower concentration in the medium. Cysteine may be slightly stimulatory to anthocyanin production. Proline permits the greatest growth when used alone and also causes a definite increase in pigment content. However, this is not strongly manifested in combination with asparagine. All these data strongly suggest that asparagine is itself inhibitory to anthocyanin synthesis in these tissues. Whether the amino acids which cause promotion of pigment synthesis do in fact reverse the inhibition caused by asparagine or simply promote synthesis in its presence cannot be determined from these experiments.

In general those amino acids which inhibited pigment synthesis in the endosperm cultures acted in a similar manner on Spirodela with one notable exception. Cystine stimulates pigment formation in the endosperm tissues, but inhibited pigment synthesis in Spirodela. Unfortunately, Thimann and Radner (11) did not test the effects of glutamic and aspartic acids.

DISCUSSION

Many of the results reported here are at variance with those of Thimann and his associates (10, 11, 12, 13). Most notable among these differences are

the responses to purine and pyrimidine analogues and riboflavine. There are several factors which could account for these differences. Admittedly, they are all speculative.

I. The endosperm cultures are isolated tissues devoid of chlorophyll in contrast to Spirodela which is a complete autotrophic plant.

II. The endosperm tissues are used in an actively growing state in these experiments while Spirodela is used in a non-growing state. This is done by floating the plants on solutions devoid of mineral nutrients.

III. The major anthocyanin pigment of Spirodela is a pelargonidin derivative (3) while that of the endosperm is a cyanidin derivative (7). Although the difference between pelargonidin and cyanidin is only one hydroxyl group on the B-ring, it has been reasonably well established that separate genes control the biosynthesis of 4′-hydroxylated aglycones (which include pelargonidin derivatives), and of 3′, 4′-hydroxylated aglycones (which include cyanidin derivatives) (2, 4). It is quite likely that the reactions controlled by these genes require different substrates, probably only subtly different.

In the discussion which follows, all three of these factors could play a contributing role to a greater or lesser degree.

Despite the inhibition induced by riboflavine in the endosperm cultures, it is still possible that the vitamin plays a role in pigment synthesis. Thimann and Radner (13) reported that anthocyanin formation in Spirodela was slightly inhibited when kept on riboflavine medium during an initial 5-day period. Within the succeeding 2 to 5 days (total 7–10 days) the pigment content frequently reached a concentration 20 to 40 % greater than the controls floated on water. Further, they state ". . . the action of riboflavine appears to require the previous formation of a photosynthetic product." It is possible that free riboflavine as such does inhibit pigment synthesis. However, after reaching a certain critical concentration within the plant, it is converted into a compound active in the formation of anthocyanin. One of the precursors of this postulated compound could be the photosynthetic product implicated by Thimann and Radner which would combine with the riboflavine.

Along with this the possibility must be considered that it is not the concentration of endogenous riboflavine that is critical, but rather the postulated photosynthetic compound. Perhaps the endosperm tissues synthesize this substance or a similar one by non-photosynthetic means to the extent that it is always present in a critical but limited concentration. Thus the endogenous riboflavine could already be bound by the available intermediate, and any additional riboflavine would be free to inhibit pigment synthesis.

Of all the amino acids tested, only four, aspartic acid, cystine, glutamic acid, and proline permitted significant increases in anthocyanin content when used alone. Of these, aspartic acid, cystine, and glutamic acid are dicarboxylic amino acids. From the inhibitions caused by asparagine and glutamine, it is tempting to draw the generalization that one of the reactions leading to anthocyanin formation requires a dicarboxylic amino acid and that the amide group in some way interferes with this reaction. Although it is true that on a molar basis, the amides would provide twice the nitrogen that the parent amino acids would, it is doubtful that the extra nitrogen per se plays a role in depressing pigment synthesis. As has been pointed out earlier in this report, a concentration of asparagine of 3.7×10^{-3} M (approx. ¼ the conc. of 1.5×10^{-2} M) does permit somewhat more pigment to be formed, but still it is far less than that produced on either casein hydrolyzate or yeast extract media.

Evidence presented in this report indicates that cytidylic acid, a pyrimidine nucleotide, and adenylic acid, a purine nucleotide enhance pigment formation to some degree. On the other hand, evidence obtained with the purine and pyrimidine analogues seems to indicate that the nucleotides do not participate directly in anthocyanin-forming reactions. If they did so participate, it would seem reasonable to expect greater inhibitions with the analogues than have been obtained with the endosperm tissues. The inhibition of growth rather than of pigment formation obtained with 2,6-diaminopurine and thioadenine indicates that interference with nucleic acid synthesis need not necessarily interfere with pigment synthesis.

Another point which should be mentioned in this connection is the central role aspartic acid plays in the biosynthesis of both purines and pyrimidines. The entire carbon chain of aspartic acid is incorporated into the pyrimidine structure (6). One of the nitrogens of the purine ring is donated by aspartic acid. Neither glutamic acid, glutamine, nor asparagine can substitute for aspartic acid in this reaction (5). Thus it is possible that some compound intermediate between aspartic acid and the full purine or pyrimidine structures (or both) occupies an important site in the synthesis of anthocyanin by the endosperm tissue cultures.

Summary

The effects of various inhibiting and stimulating agents of anthocyanin synthesis in corn endosperm tissue cultures have been determined. The strongest inhibiting agents are riboflavine, methionine, asparagine, glutamine, and valine. The strongest promoters of pigment synthesis are aspartic acid and cystine. Adenylic and cytidylic acids promote anthocyanin synthesis to some degree. Experiments with purine and pyrimidine analogues seem to indicate that the nucleotides probably do not participate directly in pigment formation. It is suggested that aspartic acid plays an important role in anthocyanin synthesis. A derivative of aspartic acid intermediate between the full purine or pyrimidine structure may be the active compound. Most of the inhibition data are at variance with those of Thimann and his associates which were obtained with Spirodela. It is suggested that differences in the metabolic states of Spirodela (a complete, green, autotrophic plant) and the endosperm

cultures (tissues devoid of chlorophyll) as well as the chemical differences between the major pigments may account for the disparity of results.

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Literature Cited

1. Edmundson, Yvette, H. and K. V. Thimann. 1950. The biogenesis of the anthocyanins. II. Evidence for the mediation of copper in anthocyanin synthesis. Arch. Biochem. 25: 79–90.
2. Geissman, T. A. and J. B. Harborne. 1955. The chemistry of flower pigmentation in *Antirrhinum majus*. IV. The albino (⁻mm⁻nn) form. Arch. Biochem. Biophys. 56: 259–263.
3. Geissman, T. A. and L. Jurd. 1955. The anthocyanin of *Spirodela obligorrhiza*. Arch. Biochem. Biophys. 56: 259–263.
4. Jorgensen, E. C. and T. A. Geissman. 1955. The chemistry of flower pigmentation in *Antirrhinum majus* color genotypes. III. Relative anthocyanin and aurone concentrations. Arch. Biochem. Biophys. 55: 389–402.
5. Lieberman, I. 1956. Involvement of guanosine triphosphate in the synthesis of adenylosuccinate from inosine 5′ phosphate. Jour. Amer. Chem. Soc. 78: 251.
6. Reichard, P. 1954. The enzymatic synthesis of ureidosuccinic acid in rat liver mitochondria. Acta Chemica (Scand.) 8: 795–805.
7. Straus, J. 1959. Anthocyanin synthesis in corn endosperm tissue cultures. I. Identity of the pigments and general factors. Plant Physiol. 34: 536–541.
8. Straus, J. Maize endosperm tissue grown in vitro. III. Development of a synthetic medium. Amer. Jour. Bot. (In press).
9. Strong, F. M. 1955. Riboflavine, folic acid and biotin. In: Modern Methods of Plant Analysis. Vol. 4. K. Paech, and M. V. Tracey, eds. Springer-Verlag. Berlin.
10. Thimann, K. V., Yvette H. Edmundson, and Babette S. Radner. 1951. The biogenesis of the anthocyanins. III. The role of sugars in anthocyanin formation. Arch. Biochem. Biophys. 34: 305–323.
11. Thimann, K. V. and Babette S. Radner. The biogenesis of anthocyanins. IV. The inhibitory effect of methionine and other sulfur-containing compounds on anthocyanin formation. Arch. Biochem. Biophys. 58: 484–497.
12. Thimann, K. V. and Babette S. Radner. 1955. The biogenesis of anthocyanins. V. Evidence for the mediation of pyrimidines in anthocyanin synthesis. Arch. Biochem. Biophys. 59: 511–525.
13. Thimann, K. V. and Babette S. Radner. 1958. The biogenesis of anthocyanin. VI. The role of riboflavine. Arch. Biochem. Biophys. 74: 209–223.
14. Underhill, E. W., J. Watkins, and A. C. Neish. 1957. Biosynthesis of quercetin in buckwheat. Can. Jour. Biochem. Physiol. 35: 219–237.

STUDIES IN STOMATAL BEHAVIOR I. EFFECTS OF MOVING AND STILL AIR ON STOMATA OF EUPATORIUM AND MENTHA[1]

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Introduction

Williams (6, 7) was the first to observe that epidermal strips taken from the area of leaf enclosed by an unswept porometer cup show much wider stomatal pores than elsewhere. Heath's experiments with Pelargonium and wheat (4, 5) showed that sweeping the porometer cup with dry or humid air caused considerable closure of the stomata as compared with the areas in still air. In a repetition of Knight's experiment on the changes in transpiration rate in relation to the leaf water content, Gregory et al (1) showed that the higher transpiration rate following a period of still air was due to wider stomatal pores and not to a higher leaf water content as suggested by Knight. They attributed the observed wide opening of stomata, possibly, to a reduction in the carbon dioxide concentration of the enclosed still air as a result of photosynthesis.

In a previous investigation (2), the author has shown that when the air stream circulating through a porometer cup attached to an illuminated cherry laurel leaf was stopped for given periods, the stomata of the enclosed leaf portion opened gradually and considerably with time. When ventilation of the porometer was renewed, the stomata showed a rapid closing movement.

The present investigation is an attempt to add to the information on the response of stomata to moving and still air.

Materials and Methods

Leaves of *Eupatorium glandulosum* H.B.K. and *Mentha spicata* L. were selected for the present investigation. The porometer cups used and the details of technique were exactly as described by the author in a previous paper (2). The rate of air flow through stomata was measured by the rate of movement of the water meniscus in the porometer tube. This was taken as an indication of the degree of opening of the stomata. In the figures, 5-minute porometer readings [as distance (cm) traveled per second × 100] were plotted against time.

Results and Discussion

To show the effect of moving and still air on the stomata of Eupatorium, the results of two of several similar experiments are presented in figure 1. In both experiments readings were begun with dry air circulating through the cups. When the flow of air was stopped, a slight unexpected stomatal closure rather than unexpected opening seemed to follow. When the dry air was circulated, there was a tendency for stomatal reopening to occur. Further cycles of still and moving air periods brought about similar effects.

The results of two representative experiments using Mentha are shown in figure 2. Here again, the readings were started with the leaf in a stream of dry air. In both experiments only small and inconsistent differences were obtained in moving vs. still air.

The present results as well as those of earlier workers (1, 5, 2) would appear to establish that plant species are not necessarily similar in their stomatal responses to moving and still air conditions. Gregory et al (1) suggested and Heath (5) strongly supported the view that the abnormally wide opening of stomata

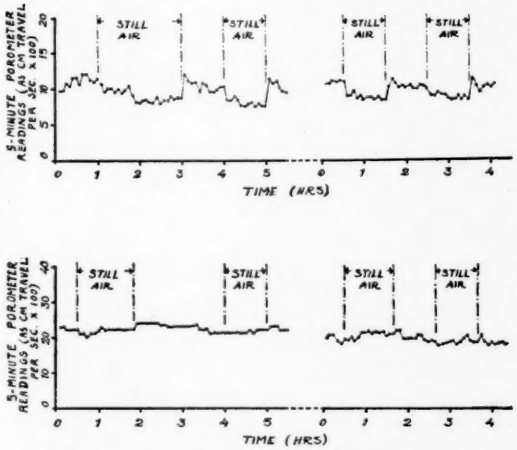

Fig. 1 (*top*). The effects of moving and still air on the stomata of *Eupatorium glandulosum*.

Fig. 2 (*bottom*). The effects of moving and still air on the stomata of *Mentha spicata*.

[1] Revised manuscript received January 28, 1960.

within an unswept porometer cup was caused by a reduction, through photosynthesis, in the carbon dioxide concentration of the enclosed air. Evidence for such a view was based on the observation of increased stomatal opening in still air with illuminated leaves of Pelargonium and wheat. Some of Heath's experimental results were, however, so curious that they imposed definite complications and attached a clear doubt to the validity of the carbon dioxide hypothesis. In particular may be cited the marked closure caused in some of his experiments with Pelargonium by change in darkness from still to moving ordinary air. In darkness, the carbon dioxide content in the porometer cup in still air would be expected to exceed the normal 0.03 % of air.

Further observations on illuminated leaves of other plant species indicated that stomatal closure or opening could not be consistently related to moving or non-moving air. Thus, in experiments with cherry laurel, the author (2) reported a rapid and very pronounced stomatal opening when one changed from moving dry normal to still air. In the present work, Eupatorium and Mentha responded differently than did cherry laurel. The stomata of Eupatorium tended to contract, while those of Mentha failed to respond appreciably when one changed from moving to non-moving air.

The dissimilarity of stomatal responses of different plant species was confirmed more rigorously by experiments shortly to be published by the author and Younis (3) with Datura, Pelargonium, Duranta, and Clerodendron. Marked and consistent differences were obtained from the four plants in response to still and to carbon dioxide-free air under similar experimental conditions. Thus, whereas changing from moving normal dry to still air brought about a very marked opening of stomata in Datura and Pelargonium, an opposite effect was found in Duranta and Clerodendron. Also, a marked opening of stomata in Datura and Pelargonium following changes to carbon dioxide-free air was not generally established in Duranta and Clerodendron. Slight indications of a possible opposite closing effect were found in most experiments with Duranta.

In view of the dissimilar experimental results so far obtained, it would seem, then, that the carbon dioxide effect cannot be generalized to include many species. This does not, of course, rule out the possibility that a carbon dioxide effect may be involved. Doubtless some of the carbon dioxide will be utilized in photosynthesis. Even in the highly responsive plants, however, the reduction of its concentration in the intercellular space system of the enclosed leaf portion and in the restricted external air supply below a critical level is not likely to be so rapid as to account for a prompt and often very pronounced stomatal opening.

Among the other factors possibly influencing the stomatal movement within porometer cups are the humidity of the restricted air and the temperature of the enclosed leaf portion. The rapid saturation of the air in the cup with water vapor will decrease water loss by decreasing the magnitude of the vapor pressure gradient from the leaf to air. This, in turn, operates in the direction of making the guard cells more turgid and the stomatal pore more widely opened. Heath's conclusion (5) excluded the possibility that the abnormally wide opening of wheat and Pelargonium when in a confined space could be due directly to the high humidity of the enclosed air. There was clear evidence, though, from his data of a humidity effect. Thus in some of his experiments with wheat a very marked stomatal closure occurred when one changed from still to dry carbon dioxide-free air. The change from still to moist carbon dioxide-free air caused (unlike the result with dry air) a very marked opening. Also, in Pelargonium the responses to still and moving air were appreciably more rapid and marked under dry conditions. Such results would strongly suggest a humidity effect.

The temperature of the enclosed leaf portion may differ from that outside. The movement of air, whether moist or dry, over the surface of that portion is likely to cool it through the increased rate of water loss by rapidly removing water vapor as it escapes. It is conceivable that the cooling effect is significant, because of the relatively large surface/volume ratio of most leaves. In still air, on the other hand, the accumulation of water vapor slows transpiration and lessens its cooling effect. Moreover, the porometer cup may act as a heat trap leading possibly to excessive heating of the leaf portion, through its interference with cooling by radiation (glass is not completely transparent to the long-wave infra red radiation).

On the grounds of the situation described here, it is suggested that carbon dioxide, humidity, and temperature may be important factors affecting directly or indirectly the movement of stomata within porometer cups. Which of these factors, under such conditions, may play a dominant role in the stomatal mechanism seems to vary with the type and structure of the plant leaf on the one hand and the outside conditions on the other. Doubtless the whole question should be investigated under far more controlled conditions than this before an unequivocal answer can be given.

Summary

The response of the stomata on illuminated leaves of Eupatorium and Mentha to moving and non-moving air has been studied. Eupatorium stomata tended to close to a greater extent in non-moving than in moving air. In moving and still air, no appreciable differences in the pore areas of Mentha stomata could be detected. These results appear inconsistent with the carbon dioxide hypothesis previously suggested to explain the abnormally wide opening of stomata of some plants within unswept porometer cups. The possibility, however, that a carbon dioxide effect may be involved was not ruled out. Other possible factors influencing the stomatal movement under such conditions have been discussed.

Literature Cited

1. Gregory, F. G., F. L. Milthrope, H. L. Pearse, and H. J. Spencer. 1950. Experimental studies of the factors controlling transpiration. II. The relation between transpiration and leaf water content. Jour. Exp. Bot. 1: 15–28.
2. Hafez, M. G. A. 1958. Effects of rosemary and thyme oil vapors on the stomata of cherry laurel. Plant Physiol. 33: 181–185.
3. Hafez, M. G. A. and M. B. Younis. 1960. Studies in stomatal behaviour. The effects of moving, still, and carbon dioxide-free air on the stomata of some plants. A'in Shams Sci. Bull., Cairo 5: (in press).
4. Heath, O. V. S. 1948. Studies in stomatal action. Control of stomatal movement by a reduction in the normal carbon dioxide content of the air. Nature 160: 179–181.
5. Heath, O. V. S. 1950. Studies in stomatal behaviour. V. The role of carbon dioxide in the light response of stomata. Jour. Exp. Bot. 1: 29–61.
6. Williams, W. T. 1947. Shock-induced stomatal movements. Nature 160: 364–365.
7. Williams, W. T. 1949. Studies in stomatal behaviour. III. The sensitivity of stomata to mechanical shock. Ann. Bot. (N.S.) 13: 309–327.

EFFECT OF SUPRA-OPTIMAL BORON LEVELS ON RESPIRATION AND CARBOHYDRATE METABOLISM OF HELIANTHUS ANNUUS[1, 2]

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A great deal of work has been done with respect to boron in plant life. However, due to the greater economic importance of boron deficiency most of the research has been focussed on the deficiency rather than the toxicity aspect.

Eaton (5) noted that boron toxicity injury was observed in a number of species at or below the substrate concentration which resulted in greatest growth. He concluded that there is considerable overlapping between injurious and beneficial effects of boron within plants. Eaton (6) noted that in a number of species there is a steep concentration gradient of boron from the center to the margin of the leaf. This may be the key to an explanation for the overlap of the two boron effects. Under normal conditions the concentration of boron may be approaching a threshold toxicity value at the leaf margins, without reaching an optimal level in the rest of the leaf. If then the major part of the leaf has normally a suboptimal boron concentration, a slight increase in substrate B might be expected to cause an increase in growth even if it also caused some toxicity effects. Thus the toxic and beneficial effects of boron may be quite distinct and their overlap may be caused only by the distribution of the element in the leaf. Another possible explanation for the overlap of the two effects is that boron toxicity results from the deleterious acceleration of reactions in which the element normally participates. This explanation has been pointed out by Gauch and Dugger (8), but they appear to favor the idea that the two effects are quite distinct and not centered on common reactions.

If either of the above explanations of the overlap is valid, a study of boron toxicity while giving much needed data on this aspect of the problem should also throw some further light on the normal functions of boron in plants.

In any comparative study it is essential to keep the number of variables as small as possible. One variable often found in boron deficiency studies is the difference in physiological age between normal and deficient plants at the time of comparison. For instance in the work of McIlrath and Palser (17) the normal tomatoes had 12.8 leaves per plant while the deficient ones had only 6.8 leaves, and the phloem was necrotic in the latter. Since this is an important variable it is possible that its effect may obscure the primary effect of boron. Thus in the work of Johnston and Dore (13) it is not certain whether the accumulation of sugars in the leaves of deficient plants was due to necrosis of the phloem or to a direct effect of boron on sugar transport. Since boron toxicity can be induced very rapidly in already mature tissue, a comparative study using plants suffering from slight boron toxicity, in place of the deficient plants, should be free from such variation in physiological development.

Although a great deal of work has been done on the essentiality of boron for plant life, there is still some controversy as to the function of boron. In recent years the majority of workers have given support to the idea that boron is involved in carbohydrate metabolism (reviewed by Gauch and Dugger, 8). Gauch and Dugger (7, 8) suggested that boron facilitates the movement of sugars in the plant by

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[2] The work was carried out while the author was the University Fellow in Plant Physiology.

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forming a more freely permeable boron-sugar complex or by joining with the cell membrane in a way that makes it more permeable to sugars. Dugger et al (4) noted that boron affected the starch-sugar balance of bean leaf discs, and with potato starch phosphorylase they showed that boron inhibits the reaction: Glucose-1-phosphate→Starch + inorganic phosphate. They concluded that boron affects the rate of translocation of sugars through the effect on the sugar-starch balance of the leaf. Spurr (23) examined the petiole of boron deficient celery prior to the onset of phloem necrosis. The walls of the phloem parenchyma were thicker and those of the collenchyma thinner than normal. This may be due to impeded translocation of sugars in the deficient plants, or to a more rapid sugar polymerisation in these plants leading to a build up of cell wall material in cells near the source but less wall material in more remote cells such as the collenchyma. Similarly Odhnoff (19) and McIlrath and Skok (18) propose that boron exerts an influence on cell development. Odhnoff (19) concludes that this is brought about by boron exerting a control over the synthesis of polysaccharides and she maintains that this is a more fundamental effect than the apparent effect of boron on sugar translocation.

From a comparative study of normal and boron deficient tomato, turnip, and cotton, McIlrath and Palser (17) concluded that the apparent effect of boron on translocation was secondary, being caused by necrosis of the phloem in boron deficient plants. This conclusion is based on the fact that the deficient cotton plants were free from phloem necrosis and had in fact a lower soluble carbohydrate level than normal cotton plants. However, their data for cotton upholds the theory of Dugger et al (4) in that the ratio of starch/sugar was higher in the deficient than in the normal plants.

Whittington (26) studying the effect of boron deficiency on germinating beans suggested that boron primarily affects the rate of cell division and not the extent of cell development. Despite a great deal of contrary evidence (19, 22, 23, 24) he renews the idea of Winfield (28) that boron is essential for pectin synthesis. It should be noted here that he is proposing the essentiality of boron in pectin synthesis, whereas Dugger et al (4) and Odhnoff (19) suggest that boron regulates a balance between sugars and polysaccharides and may even retard sugar polymerisation.

It seems therefore that despite the great deal of evidence favoring the idea that boron plays an essential role in carbohydrate metabolism, there is still doubt as to whether this is a primary role of the element.

Earlier it was pointed out that comparative studies using boron deficient and normal plants have certain disadvantages not inherent in studies with plants receiving toxic amounts of boron. The apparent overlap of the toxic and beneficial effects of boron was also mentioned. The present work was designed to perform a twofold task. First, to gain some information on the metabolism of slight boron toxicity. Second, to investigate the effect of boron on carbohydrate metabolism with particular reference to the sugar-starch balance of the plant.

Month-old sunflower plants were used, partly because they produce good boron toxicity reactions from the standpoint of localization at the perimeter, and partly because they produce fairly large amounts of leaf starch. Toxicity symptoms were induced by adding 50 ppm of boron to the culture solution 4 days prior to harvesting the plants. At this point the toxicity symptoms were confined to the extreme edge of the leaf (fig 1a).

Materials and Methods

Helianthus annuus L., var. Greystripe was used in this study. Seeds were planted in washed sterilized sand and watered with distilled water. After 1 week, seedlings were washed from the sand, selected for uniformity in height and cotyledon size, threaded into holes in corks (2 plants/cork), and held in place with plastic sponge. The roots were placed in aerated (1H) Hoagland's solution contained in half-gallon Mason jars, or in 110 liter tanks with 32 corks per tank. This solution, which contained 0.5 ppm boron, was brought to volume every day with distilled water and was replaced with fresh Hoagland's every week. In the case of the treated cultures, the fourth change of Hoagland's contained 50.0 ppm boron as H_3BO_3. By this time the plants were approximately three feet tall with seven to nine nodes.

Respiration and leaf analysis experiments were carried out when the oldest leaves of the treated plants (50 ppm B level) were just beginning to show marginal chlorosis (approximately four days after treatment). The leaves used were always taken from the second to the fifth node, in case of both treated and control plants.

Boron level and distribution were determined in leaves showing the toxicity symptoms diagrammed in figure 1a. The leaves were removed, divided into regions A, B, and C (fig 1b), and dried for 12 hours in a forced draft oven at 70° C. The carminic acid procedure of Hatcher and Wilcox (12) was used to estimate the boron content of this material.

Determination of the oxygen uptake of leaves was carried out in a Warburg respirometer, with either leaf discs cut out with a ⅜ inch cork borer (20 discs/15 ml flask), or with leaves from which the midribs had been removed (5 g of leaf material/200 ml reaction flask). The procedure of Umbreit et al (25) was used.

The respiratory quotients of treated and control leaves were determined by the direct method (Umbreit et al, 25) on leaves from which the midribs had been removed in 200 ml reaction flasks.

Because respiratory changes measured in leaves from plants grown under toxic boron levels may be a secondary response, measurements of the direct effect of boron on the respiration of the leaves were made.

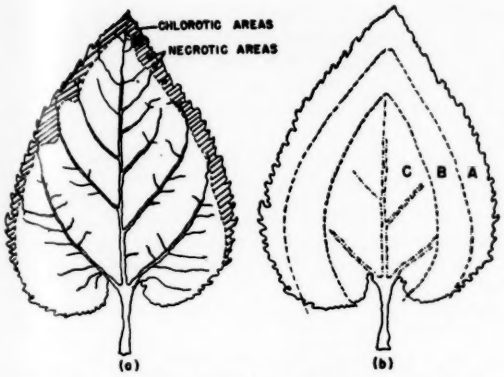

FIG. 1a. Type and location of toxicity symptoms expressed in leaves after 4 days in solutions containing 50 ppm B.

FIG. 1b. Areas for separation into regions A, B and C, for leaf analyses.

This was determined by infiltrating borate solutions into leaf discs taken from month-old sunflower plants grown in 1H nutrient. Two hundred discs were placed in each of the following boric acid solutions: 0, 150, 300, and 450 ppm boron; infiltration was achieved by repeated evacuation. Oxygen uptake values (20 discs/treatment) were determined 4 hours after the discs were placed in the boric acid solutions.

Carbohydrate and nitrogen determinations were carried out on the remaining parts of the leaves from the first three oxygen uptake determinations. The material was frozen with powdered CO_2, placed in Pyrex beakers, and then lyophilized in an apparatus similar to that described by Yamaguchi and Crafts (29). In the third experiment roots were also used. The dried material was ground in a Wiley mill to pass a 40 mesh screen.

The soluble sugars were extracted in 80 % ethanol (11). The reducing sugars in this extract were estimated by the ceric sulfate method of Hassid (10) and the total sugars by the same method following a 30 minute hydrolysis on a steam bath with 3 *N* HCl. The starch in the residue from the alcohol extraction was extracted with 52 % perchloric acid and assayed according to the method of Macready et al, (16).

The acid insoluble (5 % TCA) nitrogen of the plant material was determined by the micro-Kjeldahl method employing mercuric sulfate as the catalyst.

Starch phosphorylase was obtained by the method of Hanes (9) from the leaves of month-old sunflower plants grown in aerated Hoagland's solution. The enzyme preparation was taken up in 0.1 M citrate buffer (pH 6) and stored in the refrigerator. The reaction mixture was as follows: 1 ml of enzyme + 1 ml of 1 % starch + 3 ml of citrate buffer (pH 6) + 3 ml of water or boric acid (1,500 ppm boron). After equilibrating at 35° C, 2 ml of the appropriate glucose-1-phosphate (0.1 M, 0.08 M, 0.05 M, and 0.02 M) was added. The rate of the reaction was measured by determining the rate of appearance of inorganic phosphate. Two ml of the digest was transferred to 2 ml of ice cold 10 % TCA immediately after the G-1-P was added and twice again at 15 minute intervals. A modified Lowery and Lopez (15) method was used to determine the phosphate in the TCA extract.

RESULTS

BORON DISTRIBUTION IN LEAF: This experiment was carried out to determine whether or not there is any correlation between boron level of specific leaf areas and the expression of toxicity symptoms in these areas (5) in the case of sunflowers treated with these high dosages of boron. On a fresh weight basis there was a three-fold increase in boron from the center to the leaf edge (table I). Since the toxicity symptoms were confined to the leaf edge there was a strong correlation between boron level and the distribution of toxicity symptoms.

OXYGEN UPTAKE 1: Leaves were harvested at 3 P.M. on April 1, 1958, approximately 96 hours after treatment. The harvested leaves showed the same degree of toxicity symptoms as that depicted in figure 1a. The respiratory determinations were carried out on leaf discs from regions A and B (fig 1b) and the chemical analyses were made on lyophilized material from sections A and B.

The oxygen uptakes of both sections of the treated leaves are significantly greater than those of the control leaves (fig 2). There is no significance to the difference in oxygen uptake between A and B of the

TABLE I

BORON CONTENT OF 3 AREAS OF LEAVES (FIG 1B) AFTER 4 DAYS GROWTH IN CULTURE SOLUTION CONTAINING 50 PPM BORON *

LEAF REGION	MOISTURE %	BORON PPM IN DRY WT	BORON PPM IN FRESH WT		
			BORON	SE	CV
A	84.3	2,177	322	± 15	9.3 %
B	85.7	1,100	155	± 6	8.3 %
C	88.3	807	92	± 2	5.4 %

SE = Standard Error of Mean.
* Mean of four biological samples.

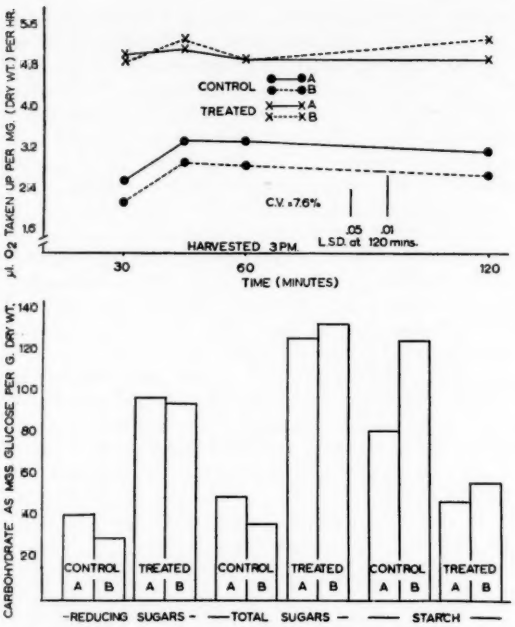

FIG. 2 (*top*). Oxygen uptake 1. The rate of oxygen uptake (Qo_2) of leaf discs from regions A and B of the treated and control leaves harvested 4 days after the addition of 50 ppm boron (H_3BO_3) to the treated cultures. These are the mean value of four replications. Differences required for significance at the 5 % and 1 % level (L S D 0.05 and 0.01) are shown.

FIG. 3 (*bottom*). The sugar and starch content of leaves from treated and control plants 4 days after treatment (as above). Mean of two replications.

treated leaves, and therefore in subsequent experiments the leaves are not divided into regions. With increase of boron in the nutrient solution there is a considerable increase in the respiration of the leaves, whether expressed on a dry weight or a protein nitrogen basis. The protein nitrogen per unit dry weight of the treated leaves is slightly lower than that of the controls (table II). Although this difference in protein nitrogen content is small it appears to be consistent as shown in later experiments.

The most striking feature of the carbohydrate analysis (fig 3) is that the level of reducing sugars and sucrose in the treated plants is three times that of the control. Whereas the starch content of the treated is only half that of the control leaves. This is in line with the observation of Dugger et al. (4) that boron inhibits starch formation. However the accumulation of soluble sugars in these treated leaves is the converse of what might be expected from the hypothesis of Gauch and Dugger (8) that boron is involved in sugar transport.

TABLE II

PROTEIN NITROGEN (5 % TCA INSOLUBLE) CONTENT OF LEAVES FROM TREATED AND CONTROL PLANTS

PLANT REGION	CONTROL		TREATED	
	A	B	A	B
Acid insol. N as mg/g dry wt	44.6	44.6	40.6	40.8

OXYGEN UPTAKE 2: Rate of oxygen uptake was determined on leaf discs which were cut randomly from the leaf blade. The treatment solution was added at 6 P.M. on April 7. Half the cultures were harvested at 6 P.M. on April 10 and the other half at 6 A.M. on April 11.

This experiment was undertaken to determine if the differences observed in the first experiment held for two periods 12 hours apart in the diurnal cycle, and if the differences could be measured when only very slight toxicity symptoms were present.

Data for the rates of respiration at 6 P.M. and 6 A.M. are presented in figure 4. The levels of respiration are approximately the same as in the previous experiment. At both harvests, however, the difference between the treated and control leaves was only significant at the 5 % level. This lowered significance is thought to be due to the fact that the boron level in the treated leaves of this experiment is lower than in the last experiment (as indicated by the lower degree of toxicity symptoms). Again the differences in respiration would be equally significant if they were expressed on the basis of protein nitrogen rather than dry weight, for the protein nitrogen is the same in both sets of leaves.

The results of the carbohydrate analysis are shown in figure 5. Differences between the treated and control leaves are noticeable, but again they are smaller than in the previous experiment (fig 3). The decline, between 6 P.M. and 6 A.M. in the amount of all three carbohydrates, was greater in the control leaves. It seems significant that despite their higher rate of respiration, the carbohydrate content (total sugars plus starch) of the treated leaves falls to 2/3 of the 6 P.M. value in the 12 hour period, whereas the control carbohydrate level decreased by a greater amount to be half of its 6 P.M. value. This suggests two possible effects of boron on carbohydrates. First, that boron slows down the translocation of carbohydrates, but this is contrary to some ideas (8). Second, that boron interferes with the synthesis of polysaccharides other than starch (23) and, therefore, a complete analysis would be required before a conclusion could be reached.

OXYGEN UPTAKE 3: This experiment was designed to answer the following questions: A. Do the rate of respiration and the carbohydrate concentration of the treated leaves remain higher than that of the control for any length of time, even when exposed to an extended dark period? (In the last experiment the decrease of carbohydrates during the dark period was less in the treated leaves than in the control

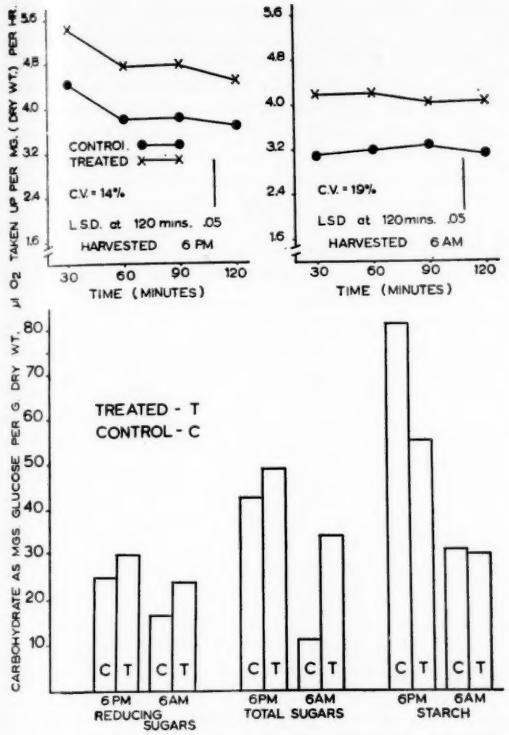

FIG. 4 (*top*). Oxygen uptake 2. The rate of oxygen uptake of leaf discs of treated and control plants. Treated April 6 with 50 ppm boron (H_3BO_3) in the nutrient culture. Harvested (a) at 6 P.M. April 10 and (b) at 6 A.M. April 11. Mean of four replications. Differences required for significance at the 5 % level (LSD 0.05) are shown.

FIG. 5 (*bottom*). The sugar and starch content of mature leaves from treated and control plants (as above). Mean value of four replications.

leaves). B. Is there a difference in carbohydrate accumulation during a light period? C. Do differences observed in the leaves hold true for other plant parts?

The plants for this experiment were grown in half-gallon Mason jars with two plants per jar. The treatment solution was added on May 28. The plants were placed in a dark chamber at midnight on May 30 and brought back into the greenhouse at noon on June 1. Leaves were harvested at noon and 8 P.M. on June 1. The leaves used in this experiment showed slightly more severe toxicity symptoms than those used in the measurement of boron distribution.

Whole leaves with midribs removed were used in the respiration determinations. At both harvest times the oxygen uptake of the treated leaves was significantly greater (1 % level) than that of the control leaves (fig 6). This high significance is thought to be related to the increased amount of boron in the treated tissue of this experiment which is indicated by the greater degree of toxicity symptoms. As in the oxygen uptake 1 experiment, the protein nitrogen is slightly lower in the treated leaves than in the controls. Therefore, the oxygen uptake difference is increased if it is expressed on the basis of protein nitrogen rather than dry weight.

Carbohydrate determinations illustrate (fig 7) that the leaves and roots of the treated plants have higher concentrations of reducing and total sugars at both harvest times. Thus despite their high rate of

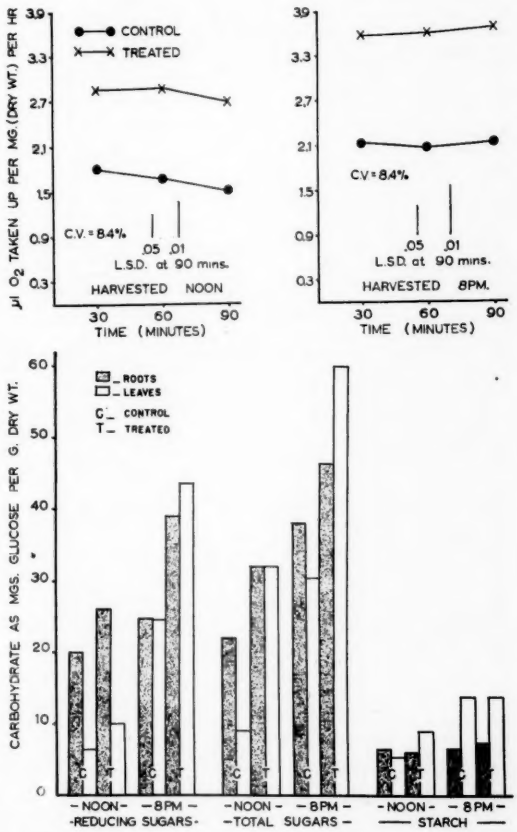

FIG. 6 (*top*). Oxygen uptake 3. The rate of oxygen uptake of leaves from treated and control plants. Treated May 28 with 50 ppm boron (H_3BO_3). Harvested a) Noon June 1 after 36 hours in the dark, b) 8 P.M. June 1 after 36 hours in the dark followed by 8 hours in the light. Mean value of four replications. Differences required for significance at the 5 % and 1 % level (LSD 0.05 and 0.01) are shown.

FIG. 7 (*bottom*). The sugar and starch content of roots and leaves from treated and control plants. Harvested 4 days after treatment. Mean value of four replications.

oxygen uptake, the treated plants maintain a higher carbohydrate (total sugars plus starch) level even after 36 hours in the dark. Since the roots also show a higher level, the accumulation of soluble sugars in the leaves cannot be due to an impeded translocation. During the period between noon and 8 P.M., the increase in the concentration of sucrose and reducing sugars is greater, and that of starch less in the treated plants. The fact that the carbohydrate concentration of the treated plants shows the greater increase, raises the question of whether the photosynthetic rate of the treated leaves is higher. It has already been noted that between noon and 8 P.M. the concentration of starch in the controls rose more than that in the treated plants (fig 7). However, in the last experiment, that of the control decreased more than that of the treated during the period between 6 P.M. and 6 A.M. (fig 5). This data suggests that the starch content of the controls fluctuates more than that of the treated plants, and that boron possibly inhibits both the synthesis and hydrolysis of starch.

The experiments described so far, raised two more questions. First, is the same respiratory substrate being utilized by treated and control leaves? Second, is the difference in respiration due to a primary effect of boron on respiration (the actual participation of boron in one or more of the reactions in the respiratory chain), or to a secondary effect (the influence of boron on the concentration of respirable substrates such as glucose)? The first question was answered by determining the R.Q.'s of the leaves, and the second by finding the effect of boron solutions on the respiration of sunflower leaf discs.

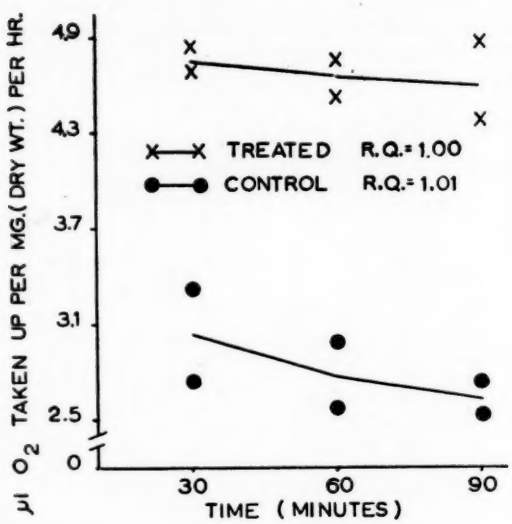

FIG. 8 (*top*). The respiratory quotient and rate of oxygen uptake of treated and control leaves 4 days after treatment with 50 ppm boron as H_3BO_3. The lines are drawn through the mean of two replicates.

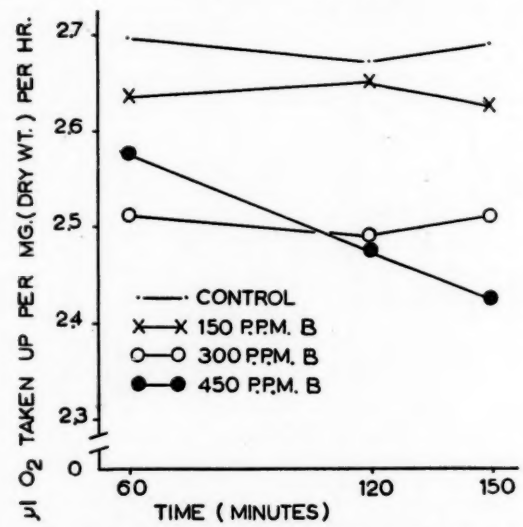

FIG. 9 (*bottom*). The rate of oxygen uptake of sunflower leaf discs 4 hours after infiltration with various boron solutions. Points represent the mean of four replications.

RESPIRATORY QUOTIENT: Plants were grown in half-gallon Mason jars. The treatment solution was added at 3 P.M. on July 4 and leaves from the second to third node were harvested at 3 P.M. on July 8. As in the last experiment, whole leaves were used in 200 ml flasks. Two replications were made and the data for the oxygen uptake and R.Q. are shown in figure 8. Since the magnitude of the difference in the respiration between treated and control leaves is approximately the same as in the previous experiments (figs 2, 4, and 6) this experiment is an acceptable representation of these three. There is no difference in the R.Q. of the two sets of leaves, thus suggesting they are using a similar type of respiratory substrate. Since the R.Q. is unity, the principal substrate should be a carbohydrate.

DIRECT EFFECT OF BORON ON RESPIRATION: Plants were grown in normal Hoagland's solution for 1 month. Discs taken from leaves of the second to fifth nodes were infiltrated with borate solutions as previously outlined.

There appears to be an inverse relationship between the rate of oxygen uptake and the level of boron in the bathing solution (fig 9). Although the differences in the respiration rates are not significant, the trend, and lack of significance were similar to that shown by Winfield (32) for tomato roots. Dugger et al, (4) found a direct relationship between boron level and the respiration of bean leaf discs. However their discs were infiltrated with 4% glucose and were incubated for 24 hours; the effect they measured may be secondary, brought about by boron's influence on carbohydrate metabolism.

EFFECT OF BORON ON STARCH PHOSPHORYLASE: Dugger et al (4) concluded that boron is a competi-

tive inhibitor of starch phosphorylase. This experiment was repeated using starch phosphorylase isolated from month-old sunflower plants. The experiment was carried out with 0 and 450 ppm B, and with a series of glucose-1-phosphate concentrations. A reciprocal plot of the velocity of the reaction and the substrate concentration is given in figure 10. The plot is approximately that for competitive inhibition. This means that boron inhibits the reaction by complexing either at the active site of the enzyme or with the glucose-1-phosphate. These possibilities are considered in the discussion.

Discussion

The treated (50 ppm boron) leaves used in this investigation showed only slight signs of toxicity injury (fig 1a) confined mostly to the extreme edges. The main part of the lamina (B and C, fig 1b) contained considerably less boron than the margin (table I) and it appeared quite normal under visual inspection. However, as shown by the first oxygen uptake experiment, the margin and the rest of the leaf did not differ significantly in their carbohydrate content or oxygen uptake. The entire leaf area was therefore used in the subsequent experiments.

The first three experiments show that the leaves of boron treated plants have a higher rate of oxygen uptake than those of the control plants. This increased rate of respiration is not caused by utilization of different substrates in the treated leaves, for both treated and control leaves have respiratory quotients of unity, indicating that carbohydrates are being catabolized in both cases. Since infiltration of leaf discs with borate solutions has no effect on their rate of oxygen uptake (fig 9) the increased rate in the leaves of the treated plants must be a secondary effect. It seems apparent from the carbohydrate analysis of these leaves that the higher rate of oxygen uptake in the leaves of the boron treated plants may result from the increased level of respirable sugars in these plants. There is some controversy in the literature as to whether boron does (4), (20), or does not (28) affect respiration. This may be due to failure of the workers to distinguish between an actual participation of boron in one or more of the reactions of the respiratory chain, and a stimulation of respiration caused by boron changing the effective concentration of respirable substrates.

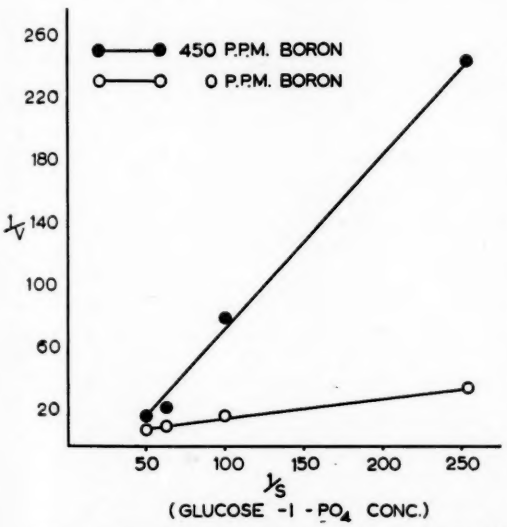

Fig. 10. Starch Phosphorylase. A comparison of the reciprocal plots of rate of reaction and substrate concentration for a reaction mixture containing 450 ppm boron (H_3BO_3) and a reaction mixture containing no added boron.

From the carbohydrate analysis the following statements can be made. In the plant there is: A. A direct relationship between boron level and the total sugar concentration; B. An inverse relationship between boron level and the starch content; C. A direct relationship between boron level and the starch plus sugar content. The accumulation of carbohydrates in the leaves of treated plants cannot have resulted from impeded translocation, for the roots also had a higher carbohydrate content than those of the controls. The data for the carbohydrate content of normal and boron deficient cotton plants reported by McIlrath and Palser (17) agrees with statements A and B but is the converse of C. However, Chkolnik et al (3) agree with C, for by spraying boron deficient plants with boron they obtained an increase in sugar plus starch. Therefore, it seems that boron when present in amounts ranging from deficient through toxic exerts an influence on the sugar-starch balance in plants.

The inverse relationship between starch content and boron level and the direct relationship between sugars and boron level may be explained on the basis of the experiment which showed that boron competitively inhibits the formation of starch from glucose-1-phosphate (fig 10), (4). If the carbohydrate level of the plant remained stable and the formation and degradation of starch was dependent only on the enzyme starch phosphorylase, competitive inhibition of this type would not account for the difference in starch level since only the rate is affected. However, since the concentration of sugars is in a state of flux, this inhibition could account for the lowered starch level. In the light there is a build up of sugars and hence of G-1-P; this has less effect on the inhibited reaction than on the rate of the control. The rate of the starch build up then is greater in the control plants, and since there is a constant feed in of G-1-P, equilibrium is not reached and at any one time the starch content of treated and control plants differ. Similarly during the dark period the fall in sugar level and hence in G-1-P will have more effect on the rate of starch formation in the controls. Since the ratio of starch/sugar is relatively higher in the controls this reduction in sugar during the dark will initially result in a more rapid hydrolysis of starch in the control plant leaves. The combination of these

two effects is that there is a more rapid fall in starch concentration in the controls. Such a mechanism explains the lowered level of starch in the treated leaves and the apparently greater fluctuations in the starch content of the controls. Dugger et al (4) postulated that boron inhibits the formation of starch by forming an unreactive glucose-1-phosphate-boron complex. The generally accepted form of competitive inhibition is where the inhibitor competes with the substrate for the active site of the enzyme. Borate complexes readily with certain polyhydroxy molecules. However there is very little complexing when the molecule has no adjacent cis-hydroxyl groups (30). Khym and Cohn (14) in studying the effect of borate on the elution of sugar phosphates from chromatograms concluded that G-1-P does not complex with borate. Thus the evidence (14, 30) suggests that boron does not inhibit the formation of starch by forming a complex with G-1-P. This means that if the inhibition is truly competitive it must be due to boron complexing at the active site of the enzyme. Complexes between boron and protein have been reported (27, 30). The effect of boron on pectin formation (23) may also be brought about by boron complexing with glycosidases involved in pectin synthesis.

In the third oxygen uptake experiment, carried out between noon and 8 P.M., the carbohydrate content of the treated leaves increased more than that of the control leaves. Baumeister (1) and Richter and Vasil'eva (21) observed a direct relationship between boron level and photosynthesis. Therefore, one possible explanation for the higher level of carbohydrates in the treated plants is that they have a more active photosynthetic system.

It has been suggested that boron is essential for protein synthesis (2). However, Beckenbach (2) suggested that the decreased protein in boron deficient plants was caused by an upset of carbohydrate metabolism in these plants. In the present investigation there was a decrease in protein nitrogen with increase in boron. This was not observed in the second oxygen uptake experiment where the toxicity symptoms were extremely slight, but it was greatest in the third experiment where toxicity was greatest. Hence it would seem that the changes in protein nitrogen content more closely follow the toxicity response than do the changes in sugars. Considerably more work must be done, however, on the relationship between boron and protein synthesis before any conclusion can be reached as to whether this is a real effect of boron on proteins or merely a response resulting from the effect of boron on carbohydrates.

The question now arises as to whether the effect of boron on the sugar-starch balance is merely an essential role of boron quite distinct from the toxic effects of the element, an essential role which is deleteriously accelerated by toxic amounts of the element, or strictly a toxic effect of boron. The similarity of the present results with those of McIlrath and Palser (17) and Dugger et al (4) would seem to rule out the last of these possibilities. The present work does not prove that this is a toxic effect of boron. However, it is possible that this is one of a number of reactions deleteriously effected by supra-optimal quantities of the element.

From the results of this investigation, together with the work of McIlrath and Palser (17) on cotton, Dugger et al (4) on starch phosphorylase, and Spurr (23) on the cell wall, the author presents the following hypothesis for one role of boron in plants. "It appears that boron performs a protective function in plants in that it prevents the excessive polymerisation of sugars at sites of sugar synthesis (it seems significant that the highest concentration of boron in the plant is in the leaves where sugars are being actively synthesized)." The inhibition of polymerisation results in an increased level of soluble sugars and apparently in an elevated pattern of metabolism as well as a higher rate of translocation.

This hypothesis is similar to that put forward by Dugger et al (4) except that these authors limited the role of boron to its participation in the reaction catalyzed by starch phosphorylase. The present author has extended it to include a general participation of boron in sugar polymerisation. Such a hypothesis will explain the effects of boron on cell development (19, 24) and on cell division (26).

It should be noted that this hypothesis is put forward as one possible role of boron in plants, for the author is of the opinion that boron may perform more than one function. For instance, it seems to be accepted (30) that during pollen germination boron forms bridges between polysaccharide molecules and so prevents the excessive stretching of the pollen cell wall. Though the element may perform a similar function at the plant meristems, the evidence suggests (4, 17, 23) that it must also perform some quite distinct function such as has been suggested in the present hypothesis.

Summary

The effect of supra-optimal quantities of boron (50 ppm) on the starch-sugar balance of month-old sunflower plants has been investigated.

Comparative studies of leaves from treated and control plants, of the same age, showed: A. The respiration rate is higher in the treated leaves, but the respiratory quotient of both sets of leaves is unity; B. The concentration of reducing sugars, sucrose, and of total sugars plus starch, is higher in the treated leaves; C. The starch content is lower in the treated leaves.

The carbohydrate content of roots of treated plants showed the same relationships as the leaves. Therefore, the increased sugar content in treated leaves cannot be the result of an impeded carbohydrate translocation.

The elevated respiratory level of the boron treated leaves is thought to be a secondary response to boron, for infiltration of normal leaf discs with borate has no effect on their respiration.

In vitro studies using starch phosphorylase showed

that boron competitively inhibits the formation of starch. It is proposed that this inhibition results from boron complexing with the active site of starch phosphorylase. It is suggested that this is only one example of a more general effect of boron on the sugar-polysaccharide balance of the plant. On the basis of this effect a hypothesis is put forward for the role of boron in plants.

Acknowledgments

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Literature Cited

1. Baumeister, W. 1943. Der Einfluss des Bors auf die Photosynthese und Atmung submersen Pflanzen. Jahr. wiss. Bot. 91: 242–277.
2. Beckenbach, J. B. 1944. Functional relationships between boron and various anions in the nutrition of the tomato. Fla. Agr. Exp. Sta. Bull. No. 395.
3. Chkolnik, M. J., M. M. Steklova, N. A. Makrova, N. V. Kovalieva, and V. N. Gretishitscheva. 1956. Role physiologique du bore chez les plantes. In: Analyse Des Plantes et Problems Des Fumures Minerales. I.R.H.O. Paris.
4. Dugger, W. M. Jr., T. E. Humphreys, and B. Calhoun. 1957. The influence of boron on starch phosphorylase and its significance in translocation of sugars in plants. Plant Physiol. 32: 364–370.
5. Eaton, F. M. 1944. Deficiency, toxicity, and accumulation of boron in plants. Jour. Agr. Res. 69: 237–277.
6. Eaton, F. M., R. D. McCallum, and M. S. Mayhugh. 1941. Quality of irrigation waters of the Hollister area of California with special references to boron content and its effects on apricots and prunes. U.S.D.A. Tech. Bull. 746.
7. Gauch, H. G., and W. M. Dugger, Jr. 1953. The role of boron in the translocation of sucrose. Plant Physiol. 28: 457–465.
8. Gauch, H. G. and W. M. Dugger, Jr. 1954. The physiological role of boron in higher plants: A review and interpretation. Univ. of Maryland Bull. A.80 (Tech.).
9. Hanes, C. S. 1940. Reversible formation of starch from glucose-1-phosphate catalysed by potato phosphorylase. Proc. Roy. Soc. (London) B 129: 174–208.
10. Hassid, W. Z. 1936. Determination of reducing sugars and sucrose in plant materials. Ind. Eng. Chem. Anal. Ed. 8: 138.
11. Hassid, W. Z. and C. C. Delwiche. 1954. Laboratory Exercises in Plant Biochemistry. Univ. of California Press.
12. Hatcher, J. T. and L. V. Wilcox. 1950. Colorimetric determination of boron using carmine. Anal. Chem. 22: 567–569.
13. Johnston, E. S., and W. M. Dore. 1929. The influence of boron on the chemical composition and growth of the tomato plant. Plant Physiol. 4: 31–62.
14. Khym, J. X. and W. E. Cohn. 1953. The separation of sugar phosphates by ion exchange with the use of the borate complex. Jour. Amer. Chem. Soc. 75: 1153–1156.
15. Lowry, O. H. and J. A. Lopez. 1946. The determination of inorganic phosphate in the presence of labile phosphate esters. Jour. Biol. Chem. 162: 421–428.
16. Macready, R. M., J. Guggaly, V. Silviera, and H. S. Owens. 1950. Determination of starch and amylase in vegetables. Anal. Chem. 22: 1156–1158.
17. McIlrath, W. J. and B. I. Palser. 1956. Responses of tomato, turnip, and cotton to variations in boron nutrition. 1. Physiological responses. Bot. Gaz. 118: 43–52.
18. McIlrath, W. J. and J. Skok. 1958. Boron requirement of *Chlorella vulgaris*. Bot. Gaz. 119: 231–233.
19. Odhnoff, Camilla. 1957. Boron deficiency and growth. Physiol. Plantarum 10: 984–1000.
20. Phillips, W. R. 1938. The effect of boron on the respiratory behaviour of tomatoes. Sci. Agr. 18: 738–740.
21. Richter, A. A. and N. G. Vasil'eva. 1941. Spraying with microelements as a way of increasing the rate of photosynthesis. Compt. rend. acad. sci. U.R.S.S. 30: 659–660.
22. Skok, J. 1958. The role of boron in the plant cell. In: Trace Elements. Academic Press, Inc., New York.
23. Spurr, A. R. 1957. The effect of boron on cell wall structure in celery. Amer. Jour. Bot. 44: 637–650.
24. Torssell, K. 1956. Chemistry of arylboric acids. IV. Effects of arylboric acids on wheat roots and the role of boron in plants. Physiol. Plantarum 9: 652–664.
25. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques. 3rd ed. Burgess Publishing Co., Minnesota.
26. Whittington, W. J. 1959. The role of boron in plant growth. II. The effect on growth of the radicle. Jour. Exp. Bot. 10: 93–103.
27. Winfield, M. E. 1945. Role of boron in plant metabolism. 2. An account of some attempts to isolate boron complexes from plant tissues. Austral. Jour. Exp. Biol. and Med. Sci. 23: 111–117.
28. Winfield, M. E. 1945. Role of boron in plant metabolism. 3. The influence of boron on certain enzyme systems. Austral. Jour. Exp. Biol. and Med. Sci. 23: 267–272.
29. Yamaguchi, S. and A. S. Crafts. 1958. Autoradiographic method for studying absorption and translocation of herbicides using C^{14}-labelled compounds. Hilgardia 28: 161–191.
30. Zittle, C. A. 1951. Reaction of borate with substances of biological interest. Advances in Enzymol. 12: 493–527.

INFLUENCE OF SALTS ON ACTIVITY OF MALIC DEHYDROGENASE FROM SPINACH LEAVES[1, 2]

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It is well established (10, 18, 19) that the respiratory rates of plant tissues containing low concentrations of salts are markedly stimulated by adding to the bathing medium any one of a variety of salts. In view of this fact and of the limited information available on the biochemical role of certain essential mineral ions in metabolism it seems that any detailed information concerning the influence of the ionic environment on the activities of important respiratory or other plant enzymes may be of considerable physiological interest.

There is considerable information in the literature indicating effects of the ionic environment on the activity of both particulate and soluble enzyme preparations. It has been reported (13) that inorganic salts of both univalent and divalent cations markedly stimulated the activity of particulate cytochrome oxidase. Honda et al (6) have shown that the rate of oxidation of reduced diphosphopyridine nucleotide (DPNH) catalyzed by washed mitochondria from beet root (*Beta vulgaris* L.) was increased by adding certain salts to the assay medium. The activity of glucose-6-phosphate dehydrogenase was reported to be increased as much as six-fold by low concentrations of certain anions (20). The ionic environment markedly affected the Michaelis constant (K_M) of this enzyme with respect to both glucose-6-phosphate and TPN. Massey (12) reported that sulfate, selenate, arsenite, citrate, borate, phosphate, and arsenate, in increasing order of effectiveness, activated salt-free fumarase and altered the pH of optimal activity while chloride, bromide, thiocyanate, and iodide in increasing order of effectiveness, inhibited the activity of this enzyme. The extent of inhibition was markedly dependent on pH. Zelitch (29) has reported that the rate of reduction of hydroxypyruvate as catalyzed by crystalline glyoxylic acid reductase was markedly increased by various anions and that the magnitude of activation was not the same at different pH levels. Eichel (5) reported that DPNH oxidase activity of cell-free homogenates of *Tetrahymena pyriformis* was increased two- to three-fold when phosphate was used with tris(hydroxymethyl)aminomethane (Tris) buffer. Other compounds such as KCl, NaCl, K_2SO_4, or Na_2HAsO_4 replaced phosphate in systems buffered with Tris.

Theorell et al (23) reported that chloride, bromide, sulfate, nitrate, formate, and acetate markedly reduced the activity of the alcohol dehydrogenase system. Walaas and Walaas (27) found that certain anions inhibited the activity of D-amino acid oxidase from pig kidney.

A method for purifying malic dehydrogenase from animal tissue has been described by several authors (4, 21, 25, 28). The occurrence of this enzyme in higher plant species has been reported by a number of workers (1, 2, 3, 11, 24, 26); however, no appreciable purification from higher plant species has been accomplished. To the authors' knowledge, the influence of the ionic environment on the activity of malic dehydrogenase has not been investigated. It is the purpose of this paper, therefore, to describe briefly the purification and properties of malic dehydrogenase from spinach leaves and to report detailed studies of the influence of a variety of salts on the activity of preparations of different purity.

MATERIALS AND METHODS

PREPARATION OF SPINACH ACETONE POWDER: Fresh spinach was obtained from local markets and thoroughly washed with water to remove any foreign particles. Laminae of leaves were removed from midribs, frozen with CO_2 snow, and then macerated in a Waring Blender with ten weights of acetone at −20° C. Liquid was removed by vacuum filtration and the residue again was macerated with five weights of cold acetone. The liquid was removed by vacuum filtration and the procedure was repeated using five weights of cold acetone. The powder was transferred to a suction flask and dried by evacuation for 2 hours at 0° to 4° C and then stored at −15° C in an evacuated flask containing anhydrous calcium sulfate.

PREPARATION OF HIGHLY PURIFIED MALIC DEHYDROGENASE: The enzyme was purified by a method involving ammonium sulfate precipitation, heat sensitization, ethanol fractionation in the presence of ammonium sulfate, chromatography on a cellulose-calcium phosphate gel column, and finally, electrophoresis on strips of filter paper. The latter step, however, did not yield sufficient enzyme for extensive experimentation. Details of a representative purification procedure are described below; the results of the experiment are summarized in table I. All preparative procedures, unless otherwise indicated, were carried out at a temperature ranging between 0° to 4° C.

STEP I. PREPARATION OF CRUDE EXTRACT: One hundred and eighty grams of acetone powder of spinach leaves were mechanically stirred for 30 minutes

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with 20 weights of 0.05 M potassium phosphate buffer at pH 7.4. The suspension was centrifuged for 10 minutes at 800 × G and the supernatant fluid was decanted. The precipitate was reextracted for 30 minutes with 8 volumes (based on initial weight of the acetone powder) of 0.05 M potassium phosphate buffer and the suspension was centrifuged again as previously described. The supernatant fluids from the two extractions were combined, filtered through four thicknesses of cheesecloth and centrifuged for 20 minutes at 10,000 × G.

STEP II. AMMONIUM SULFATE FRACTIONATION AND HEAT SENSITIZATION: Sufficient solid ammonium sulfate was added slowly with mechanical stirring to the supernatant fluid from Step I to bring the concentration to 30 % of saturation (176 g/l). The suspension was centrifuged for 10 minutes at 10,000 × G and the precipitate discarded. The concentration of ammonium sulfate in the supernatant liquid was increased to 70 % of saturation by adding 273 g ammonium sulfate per liter of extract. The precipitated suspension was heated slowly (1–1.5° C/min) with rapid stirring to 55° C and held at this temperature for 15 minutes. The suspension was cooled rapidly in an ice bath to 0° C and centrifuged for 10 minutes at 10,000 × G. The supernatant fluid was decanted and discarded and the precipitate which contained the enzyme was dissolved in 12.5 % of the original volume of 0.05 M potassium phosphate buffer at pH 7.4. After thoroughly stirring the suspension of precipitated protein in the buffer solution, the denatured protein was removed by centrifugation. The specific activity of the soluble extract was changed very little by the heat denaturation but the efficiency of later purification steps apparently was increased by this treatment.

STEP III. ETHANOL FRACTIONATION IN PRESENCE OF AMMONIUM SULFATE: Sufficient ammonium sulfate was added slowly with mechanical stirring to the extract from Step II to bring the concentration to 20 % by weight (sp gr, 1.118). Ethanol (95 %), previously cooled to −20° C, was added slowly with mechanical stirring in the proportion of 1.1 ml for each 1.0 ml of extract. The extract was cooled by means of an ice-salt mixture from 0° C at the beginning of the ethanol addition to −15° C at the end of the ethanol addition. The suspension was allowed to stand 15 minutes after adding the ethanol and the precipitated protein was collected by centrifuging at −18° C for 15 minutes at a force of 10,000 × G. The supernatant fluid was decanted and 95 % ethanol at −20° C was added slowly with mechanical stirring at the rate of 0.55 ml per ml of supernatant solution. After 15 minutes at −15° C the suspension was centrifuged for 15 minutes at 10,000 × G at a temperature of −18° C. The supernatant fluid was discarded and the precipitate was dissolved in a volume of water equal to 10 % of the volume of the extract from Step II. After centrifugation to remove denatured protein the extract was dialyzed for 4 hours against 4 liters of 0.001 M potassium phosphate buffer at pH 7.4. This was necessary in order to remove traces of ethanol and ammonium sulfate which possibly might have interfered with the chromatographic procedure.

STEP IV. COLUMN CHROMATOGRAPHY: A column (5.25 cm × 27 cm) was prepared by adding a mixture containing 144 g cellulose powder, 720 ml of calcium phosphate gel (10 mg solids per ml), and 720 ml of water to the chromatography tube and allowing the excess water to drain. The column was washed with 1 liter of water under 3 pounds pressure and then the extract from Step III was placed on the column by means of pressure. An additional 300 ml of water was washed through the column after adding the extract. The protein was eluted from the column with ammonium sulfate by the gradient elution technique essentially as described by Palmer (16). The reservoir used for the elution contained 4 % ammonium sulfate at pH 8.0 and initially the mixing chamber contained 400 ml of distilled water. The reservoir

TABLE I

PURIFICATION OF MALIC DEHYDROGENASE FROM AN ACETONE POWDER OF SPINACH LEAVES*

STEP	VOLUME	TOTAL UNITS × 10^{-3}	PROTEIN	SPECIFIC ACTIVITY	RECOVERY
	ml		*mg/ml*	*Units/mg protein*	*%*
1. Crude extract of acetone powder	2,560	17,400	7.2	944	
2. Ammonium sulfate fractionation and heat sensitization					
a. Supernatant from 30 % ammonium sulfate precipitation	2,600	20,800	5.7	1,403	119
b. Heat sensitization after 70 % ammonium sulfate saturation	343	15,800	34.0	1,353	91
3. Ethanol fractionation	135	5,400	3.4	11,760	31
4. Chromatography (combined fractions 35 through 43)	170	999	0.17	35,000	5.8
5. 50–65 % ammonium sulfate fraction	5	600	1.3	92,300	3.5

* The enzyme was purified from 180 g of acetone powder. See text for details of the procedure.

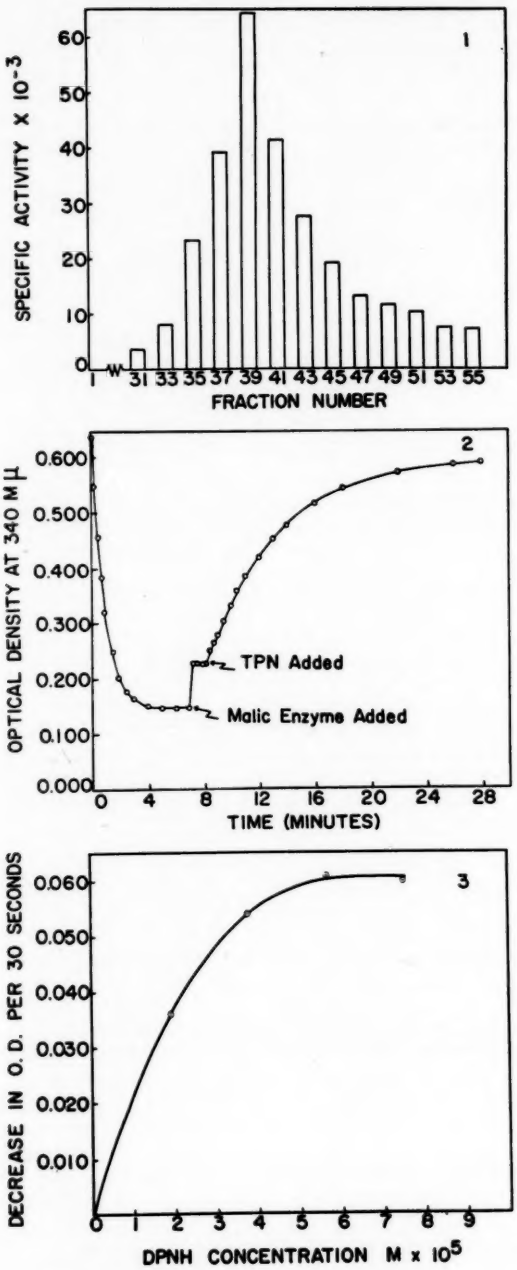


FIG. 1. Calcium phosphate gel chromatography of malic dehydrogenase. The extract was placed on the column and eluted with ammonium sulfate by the gradient elution technique. The effluent was collected in 20 ml increments and every second increment was assayed for protein and enzyme activity.

was placed approximately four feet above the column giving sufficient head to force the eluting agent through the column at the rate of about two milliliters per minute. The effluent was collected in 20 ml increments; every second increment was assayed for enzyme activity and protein concentration. The results of a typical experiment are shown in figure 1. The nine fractions with the highest specific activities were combined for further purification.

STEP V. SECOND AMMONIUM SULFATE FRACTIONATION: Sufficient solid ammonium sulfate (313 g/l of extract) was added to the combined fractions from Step IV to bring the concentration to 50 % of saturation. After standing for 15 minutes and centrifugation to remove the precipitated protein the supernatant extract was brought to 65 % saturation with ammonium sulfate by adding 101 g per liter of extract. The suspension was centrifuged and the precipitate which contained the active enzyme was dissolved in a volume of 0.05 M potassium phosphate buffer (pH 7.4) equal to approximately 3 % of the volume of the combined fractions from Step IV. Paper electrophoresis (as described in Step 6) indicated that this extract contained only two protein components in sufficient quantities for detection. Less than 0.03 μg of protein was required for the assay of this preparation and the specific activity of it was 92,300 (table I).

STEP VI. PAPER ELECTROPHORESIS: On each of three strips of Whatman No. 3 MM filter paper saturated with barbital buffer was placed in a line, 0.2 ml of the extract from Step V. The chamber of the electrophoresis unit (E-C Apparatus Co., New York, N. Y.) was filled with barbital buffer at pH 8.6 having an ionic strength of 0.1. The instrument containing the paper strips was placed in a cold room at 0° to 2° C and a potential of 200 volts was applied for 20 hours. At the termination of this period one of the three strips was dried at 110° C and observed under an ultraviolet light (3,660 A) which disclosed two distinct fluorescing bands. The distances from the origin to the center of the bands, which moved toward the anode, were 3.5 and 7.0 cm, respectively, and the widths of the bands were 2 cm and 1.5 cm, respectively. Sections were cut from the two undried strips at locations corresponding to the location of

FIG. 2. Identification of malate as the reaction product. The cuvette contained 50 micromoles glycylglycine buffer, pH 7.4, 0.33 micromole DPNH, 1.5 micromoles oxaloacetic acid, 1.25 micromoles $MnCl_2$, 2.2 ml water and 37 units malic dehydrogenase from Step V. Seven minutes after the malic dehydrogenase reaction was initiated 28 units of malic enzyme were added and then after another minute 0.5 micromole TPN was added.

FIG. 3. Effect of DPNH concentration on enzyme activity. The standard assay procedure was used with the exception that DPNH was varied as indicated. Undialyzed enzyme from Step VI was utilized.

the bands on the dried strip. The protein was eluted from the sections with 3 ml of 0.05 M potassium phosphate buffer and the eluate was assayed. The malic dehydrogenase activity of the eluted band nearest the origin (3.5 cm) was approximately 20 times that of the eluate from the band located 7.0 cm from the origin. Although the protein concentration of the eluates was too dilute to be determined by analytical methods, it was noted that the intensity of the fluorescence of the two bands under ultraviolet illumination was approximately equal. Based on the assumption that 50 % of the protein in the extract from Step V was malic dehydrogenase the specific activity of the eluate of the band nearest the origin was roughly calculated to be 185,000.

PREPARATION OF PARTIALLY PURIFIED MALIC DEHYDROGENASE: In some of the experiments reported an extract was utilized which was obtained by partial purification of the crude extract. For this procedure the crude extract (Step I) was brought to 50 % saturation with ammonium sulfate, allowed to stand 10 minutes and centrifuged for 10 minutes at 15,000 × G. The precipitated protein was discarded and the supernatant solution was brought to 65 % saturation with ammonium sulfate. After standing 10 minutes the suspension was centrifuged and the precipitated protein was redissolved in a third the original volume of 0.025 M glycylglycine buffer adjusted with solid Tris to pH 7.4. This procedure resulted in a five-fold purification with a recovery of about 55 % of the activity in the crude extract. This extract was dialyzed for 16 hours at 0° to 4° C against 0.001 M glycylglycine (pH 7.4) to remove the remaining ammonium sulfate. The dialyzed enzyme lost its activity rather rapidly during storage. This protein fraction, hereafter referred to as partially purified enzyme, was used in all experiments concerning the effects of salts on activity unless otherwise indicated. In all cases the results obtained with highly purified extracts exhibited trends similar to those obtained with partially purified extracts.

PREPARATION OF MALIC ENZYME: Crude malic enzyme was prepared according to the procedure of Ochoa (15). Partial purification was accomplished by adding slowly with stirring sufficient solid ammonuim sulfate to bring the extract to 35 % saturation. After standing 10 minutes, the suspension was centrifuged 15 minutes at 10,000 × G. The supernatant fluid was decanted and brought to 55 % ammonium sulfate saturation. Precipitated protein was collected by centrifuging at 10,000 × G and redissolved in a third the original volume of 0.05 M potassium phosphate buffer, pH 7.4. Ammonium sulfate fractionation yielded a four-fold purification.

CHEMICALS: Reduced diphosphopyridine nucleotide (DPNH) of 90 to 95 % purity, reduced triphosphopyridine nucleotide (TPNH) of 95 to 100 % purity, oxidized triphosphopyridine nucleotide (TPN) of 95 to 100 % purity, oxaloacetic acid (OAA), and glycylglycine were obtained from the Sigma Chemical Co., St. Louis, Mo. The concentrations of DPNH and TPNH solutions were determined with a Beckman DU spectrophotometer by the extinction coefficient of 6.24×10^6 cm^2 × moles^{-1} at 340 mμ (7). Malic acid was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Acetone used in the preparation of the acetone powder was of commercial grade quality. Barbital buffer (pH 8.6) having an ionic strength of 0.1 was prepared by dissolving 29.43 g sodium barbital, 19.43 g sodium acetate, and 11.71 g anhydrous sodium acetate in 200 ml of 0.1 *N* HCl and adding sufficient water to adjust the final volume to 3 liters. Other chemicals used were of reagent grade and were obtained from commercial sources.

Sodium salts of the organic acids were prepared by neutralizing the acids to pH 7.4 with NaOH. At this pH the salts were more than 98 % dissociated and the ionic strengths of these solutions were calculated on the assumption that dissociation was complete.

STANDARD ASSAY PROCEDURE: Individual assays were carried out at room temperature in a final volume of 3 ml essentially as described by Ochoa (14). Each complete reaction mixture in a spectrophotometer curvette contained the following constituents: 50 micromoles glycylglycine buffer adjusted to pH 7.4 with solid Tris; 0.19 micromoles DPNH; 0.76 micromoles oxaloacetic acid adjusted to pH 7.4 with Tris, and sufficient enzyme to give a decrease in optical density of 0.01 to 0.03 per 30 seconds. In studies of the influence of salts on activity the desired salt concentration was obtained by adding appropriate amounts of 1.0 *N* salt solutions. The reaction was initiated by adding oxaloacetate. Under these conditions the rate of reduction of oxaloacetate was approximately linear for the first minute. Activity of the enzyme is expressed as the decrease in optical density at 340 mμ during the interval between 15 and 45 seconds after initiating the reaction. One unit of enzyme activity is defined as that amount which will cause an optical density decrease of 0.01 per minute under the previously described conditions. Specific activity is expressed as units per mg of protein. Exposure of enzyme extracts to a temperature of 100° C for 1 minute destroyed activity.

OTHER ASSAY PROCEDURES: In assaying the oxidation of malate to oxaloacetate by malic dehydrogenase the experimental cuvette contained the following constituents in a final volume of 3 ml: 25 micromoles glycine buffer adjusted to pH 9.2 with NaOH, 0.19 micromoles DPN, 44 micromoles malate adjusted to pH 9.2 with NaOH, enzyme (15–20 μg protein) and the desired concentration of salt. The reaction was initiated by adding malate, and activity was determined by the increase in optical density at 340 mμ between 15 and 45 seconds after starting the reaction.

The concentration of protein was determined with Folin's reagent (9).

Results

Identification of Reaction Product: Evidence that L-malic acid is produced concurrently with DPNH oxidation is presented in figure 2. After allowing the malic dehydrogenase reaction (fig 2) to reach equilibrium a partially purified preparation of malic enzyme from pigeon liver was introduced into the reacting mixture. The initial increase in optical density following the addition of malic enzyme was due to absorption by the extract. There was no further change in optical density until the addition of TPN and then the optical density increased until it reached a level slightly below the initial optical density. In an experimental cuvette containing TPN, malic enzyme, and all components of the malic dehydrogenase reaction except DPNH there was no optical density change until L-malate was introduced.

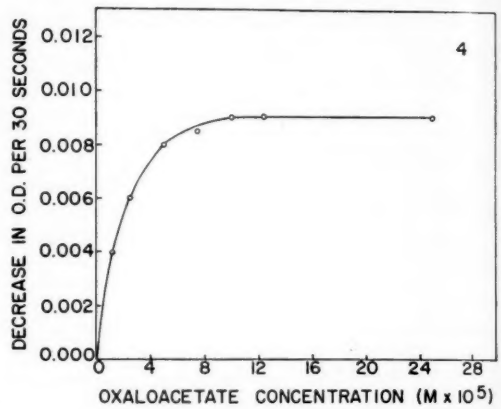
During the initial malic dehydrogenase reactions 0.227 micromoles of malate was formed. Malic enzyme subsequently catalyzed the oxidation of 0.180 micromoles of malate, or 80 % of the malate formed in the malic dehydrogenase reaction. Undoubtedly a greater proportion of the malate would have been utilized if the reaction had been allowed to proceed for a greater length of time; however, complete utilization was not expected with the concentrations of TPN added in the reaction mixture. Since malic enzyme is specific for L-malate (15), the evidence indicates that L-malate must be the product of the malic dehydrogenase reaction.

Substrate Saturation: The influence of DPNH concentration on the velocity of the reaction is shown in figure 3. The Michaelis constant (K_M) with respect to DPNH was estimated from this curve to be 1.5×10^{-5} M. A sample of the enzyme extract from Step VI of the purification also was assayed with TPNH as the electron donor. When assayed with 0.19 micromoles of TPNH in the assay mixture the rate of the reaction was 1.5 % of that obtained with a comparable concentration of DPNH.

The concentration of oxaloacetate required to saturate the enzyme in the presence of optimum concentrations of DPNH was approximately 1×10^{-4} M (fig 4). The K_M with respect to oxaloacetate which was estimated from figure 4 is 1.8×10^{-5} M. When nine units of enzyme were tested under the standard assay conditions with the exception that either pyruvate, glyoxalate or alpha-ketoglutarate instead of oxaloacetate was added at concentrations up to 1.3×10^{-3} M, no activity was exhibited.

pH Optimum: The effect of pH of the medium on the activity of the enzyme is shown in figure 5. Maximum activity with the enzyme preparation from Step V occurred at pH 7.0 to 7.4 when sodium phosphate buffer was used. Enzyme activity decreased rapidly below pH 6.2 and above pH 7.4. As indicated by subsequent experiments the activity of malic dehydrogenase is influenced by the ionic concentration of the medium. The determination of optimum pH, therefore, is complicated by the ionic strength of the buffer. The fact that the ionic strength of the medium increases with increasing pH values when phosphate buffers of equal molarity are used places some degree of uncertainty on the values given for optimum pH. For this reason it is possible that different pH optima may be produced by different buffers or even by the same buffer at different concentrations.

Influence of Inorganic Salts on Activity: The enzyme was assayed in the presence of different concentrations of several inorganic salts and the results of these experiments are presented in figure 6. These curves were obtained with partially purified extracts. Similar curves were obtained with extracts from Step V of the purification procedure. In general, adding sodium salts of different anions at concentrations up to 0.04 N resulted in striking increases



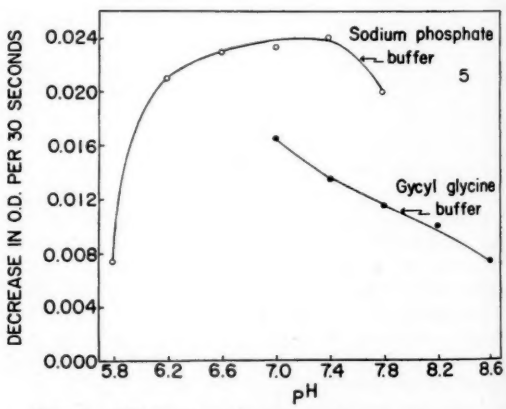


Fig. 4. Effect of oxaloacetic acid concentration on enzyme activity. The standard assay procedure was used with the exception that OAA was varied as indicated. Undialyzed enzyme from Step VI was utilized.

Fig. 5. pH Optimum of spinach malic dehydrogenase. The standard assay medium was used with variation in the pH of the buffer (concs of 0.0133 M) as indicated. Glycylglycine buffer was adjusted to the desired pH with solid Tris.

in enzyme activity. Sodium phosphate produced a two-fold increase in activity with a broad optimum ranging from 0.03 to 0.08 *N*. A stimulation of about the same magnitude also was produced by adding $NaNO_3$ but maximum activity was observed within a very narrow range of salt concentrations. Maximum activity was obtained when $NaNO_3$ at a concentration of 0.04 *N* was included in the assay mixture. The magnitude of activation by Na_2SO_4 was considerably less than that of the other inorganic salts. Concentrations of this salt ranging between 0.025 and 0.04 *N* resulted in an increase in activity of about 65 %.

The chloride salt of sodium produced an effect on enzyme activity different from that of the other anion salts of sodium. As the NaCl concentration was increased the enzyme activity increased until a maximum was reached at a concentration of approximately 0.04 *N*. Above this concentration activity decreased with increasing increments of salt until a concentration near 0.05 *N* was reached. Further increases in NaCl concentration produced another increase in enzyme activity with a second peak in the curve appearing when a concentration of approximately 0.07 *N* NaCl was utilized. At concentrations greater than 0.07 *N* activity decreased as the salt concentration was increased.

The addition of chloride salts of univalent cations other than sodium resulted in increases in enzyme activity with trends similar to those obtained with NaCl (fig 7). In general, maximum activity was observed at concentrations of approximately 0.04 and 0.07 *N* while a lower rate of activity was exhibited at Cl^- concentrations between these two optima. To determine the reproducibility of the two peaks apparent in the Cl^- activation curve, the enzyme was assayed in the presence of 0.040, 0.058, and 0.075 *N* KCl, respectively. The determination at each salt concentration was repeated five times and the mean O.D. changes per 30 seconds with 0.040, 0.058, and 0.075 *N* salt were 0.029, 0.026, and 0.028, respectively. The difference between means required for statistical significance at the 1 % level was 0.002.

When different extracts were assayed with a series of concentrations of either NaCl or KCl it was observed that the magnitude of stimulation from a salt concentration of 0.04 *N* varied little from two-fold. The magnitude of stimulation obtained from adding sufficient salt to produce the second peak (0.070–0.075 *N*), however, varied from two-fold to three-fold. The concentrations of salt producing maximum activity were relatively constant among the experiments with different extracts.

The influence of various halide salts on enzyme activity is illustrated by the data presented in figure 8. Similar curves were obtained regardless of whether Na^+, K^+, Li^+, or NH_4^+ was utilized as the balancing cation. A stimulation of approximately two-fold was produced by adding either NaBr or NaI at concentrations of approximately 0.033 *N*. Enzyme activity decreased rather rapidly as the concentration of these salts was increased above 0.033 *N*. Increasing concentrations of these halides, however, failed to produce a second maximum in activity and in this respect behaved differently from chloride and fluoride salts. Fluoride salts of univalent cations produced a series of maxima. In several experiments with salts of this anion, maxima were observed at concentrations of 0.033 to 0.04 *N*, 0.06 to 0.07 and about 0.1 *N*. Activity decreased slowly as the fluoride concentration was increased from 0.1 *N* to 0.2 *N*.

In an effort to determine whether or not the two activity maxima obtained from adding NaCl were induced by total salt concentration or by a specific ion species, a series of experiments was conducted in which NaCl concentrations were varied at different levels of $NaNO_3$ (fig 9). In the absence of $NaNO_3$ an inflection in the curve was observed with a concentration of NaCl of about 0.035 *N* and a maximum was produced at a concentration of 0.075 *N*. These are the concentrations which normally gave maximum activity (figs 6, 7, and 8). In the presence of 0.017 *N* $NaNO_3$ a less prominent inflection in the curve was observed at a concentration of NaCl of about 0.025 *N* while the concentration producing the second maximum was 0.075 *N* as previously observed. When the influence of NaCl concentration on the enzyme activity was determined in the presence of 0.033 *N* $NaNO_3$, activity measurements without NaCl were relatively high and these decreased as the NaCl concentration was increased to 0.025 *N*. With higher concentrations of NaCl the activity again increased to a maximum at 0.075 *N*. Although the second peak in this curve was not prominent, it was consistently reproduced in other experiments. These curves indicate that the location of the first optimum depends upon the total salt concentration while the location of the second optimum is dependent upon the concentration of NaCl.

In attempting to elucidate the mechanism by which salts stimulate malic dehydrogenase activity it was considered that knowledge of the influence of salts on the K_M of the enzyme for OAA and DPNH would be of value. Plotted in figures 10 and 11 according to the method of Lineweaver and Burk (8) are data which were obtained when the concentrations of either OAA (fig 10) or DPNH (fig 11) were varied in the presence of different levels of NaCl. Failure of the projected plots to intersect at infinite substrate concentration indicates that the effect of NaCl is not overcome with high concentrations of substrate. The deviation from linearity of the lower parts of the plots in figure 10 is due to inhibition of the enzyme by OAA. These data suggest that the action of NaCl is upon some point other than the active site of the enzyme.

The K_M values for malic dehydrogenase with respect to OAA and DPNH were calculated from figures 10 and 11. The values with respect to OAA in the presence of no salt, 0.033 *N* NaCl and 0.067 *N* NaCl were, respectively; 2.88×10^{-5}, 4.10×10^{-5}, and 4.45×10^{-5}. The K_M values with respect to DPNH in the absence of salt and in the presence of 0.033 *N* NaCl were, respectively; 1.24×10^{-5} and 2.44×10^{-5}.

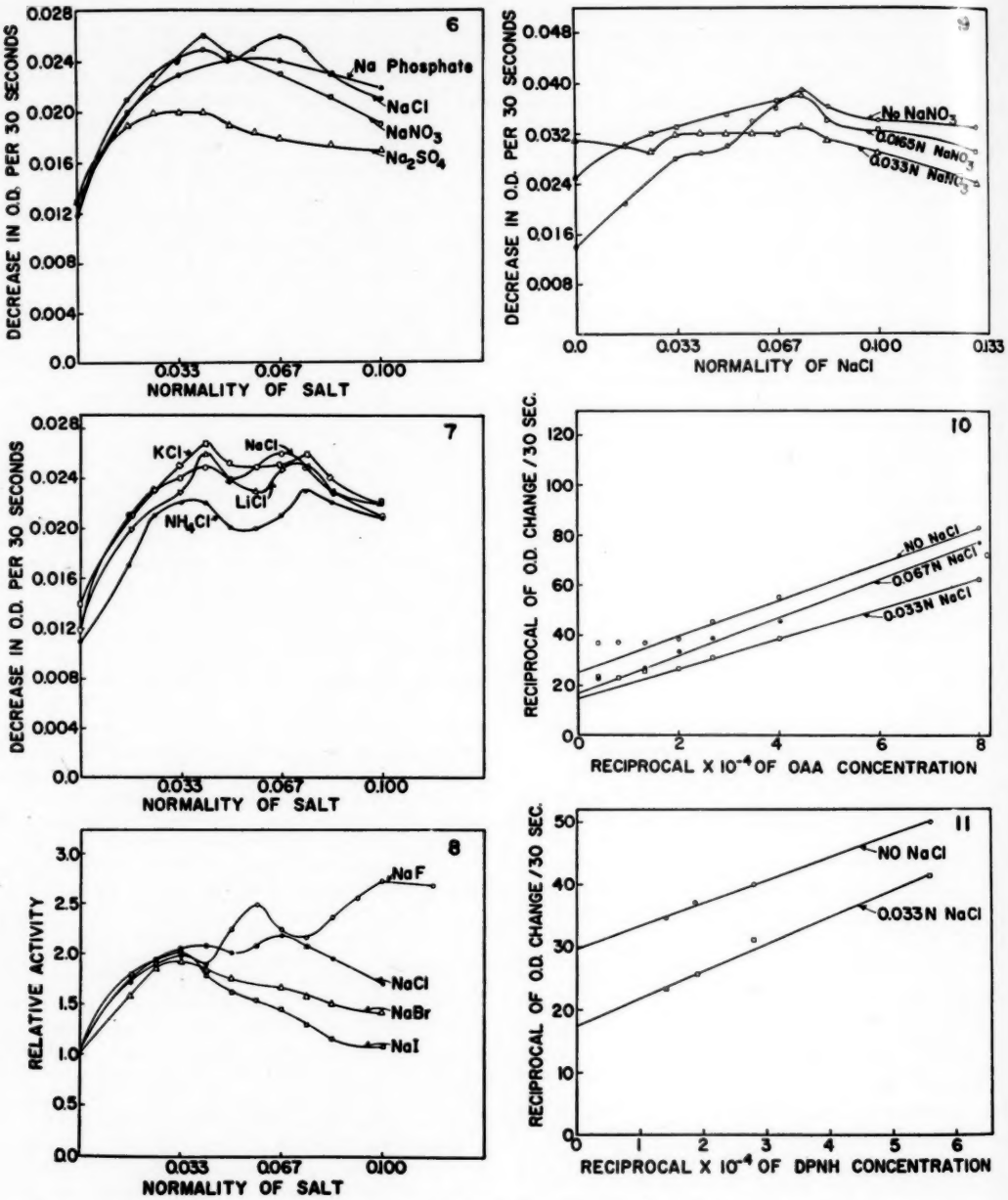


FIG. 6. Effect of concentration of various salts on malic dehydrogenase activity. The standard assay procedure was used with variation in the salt contents as indicated. The enzyme consisted of approximately six μg of protein from the partially purified extract.

FIG. 7. Effect of chloride salts of univalent cations on malic dehydrogenase activity. The standard assay procedure was used with variation in concentration of chloride salts as indicated. The enzyme extract contained approximately six μg of protein from the partially purified enzyme. Experiments with the various salts were carried out with separate dilutions of the partially purified extract.

FIG. 8. Effect of sodium salts of various halides on malic dehydrogenase activity. The standard assay procedure was used with variation in concentration of salts as indicated. Relative activity is the activity in the presence of

Chloride salts of divalent cations produced maximum activity at lower normalities than those of univalent cations. The differences, however, were not marked when activity was plotted against the ionic strength of the medium. From the results presented in figure 12 it is apparent that 2–1 electrolytes produced maximum activity at about the same ionic strength that gave maximum activity with the 1–1 electrolytes. Unlike the chloride salts of univalent cations, the chloride salts of Mg and Mn failed to produce two peaks in the stimulation curve. Curves similar to those of $MgCl_2$ and $MnCl_2$ were obtained by use of $CaCl_2$ or $BaCl_2$.

Influence of Organic Salts on Activity: The enzyme was assayed in the presence of different concentrations of sodium salts of a series of organic acids. Three-fold stimulation was produced by a concentration of 0.1 *N* sodium acetate or 0.1 *N* sodium formate (fig 13). As shown in figure 13 sodium salts of succinic, tartaric, and citric acids produced maximum activities at the same ionic strength which gave maximum activity with acetate or formate. The magnitude of stimulation at optimal concentrations of the organic salts decreased with increasing valency of the acid. The magnitude of stimulation was approximately 3-, 1.9-, and 1.5-fold for the univalent, divalent, and trivalent acids, respectively.

Influence of pH on Salt Activation: A series of experiments was conducted to determine whether or not the pH of the assay medium influenced the magnitude of salt activation. For these experiments phosphate buffer was used at a final concentration of 6.7×10^{-3} M. In figure 14 are presented the results of experiments in which the influence of the concentration of NaCl on enzyme activity was determined at pH values of 6.2, 7.0, and 7.8. From these curves it is apparent that concentrations of NaCl ranging from 0.075 to 0.080 *N* resulted in maximum activity at each of the various pH levels. The magnitude of the salt stimulation differed, however, at the three pH levels tested. The increases in activity resulting from the addition of salt were 163 %, 125 %, and 66 % at pH levels of 6.2, 7.0, and 7.8, respectively. In these experiments, as noted previously, NaCl produced only one definite peak in the stimulation curve. There are definite inflections in the curves, however, at concentrations near those that normally resulted in peaks (figs 6, 7, and 8).

Influence of Salts on Malate Oxidation: It was of interest to determine whether the salt activation of the reaction catalyzed by malic dehydrogenase occurred in both the forward and reverse directions. It is apparent from the results presented in figure 15 that the rate of enzymic oxidation of malate to OAA also is stimulated by adding salts to the assay medium. In general, optimal concentrations of salts for malate oxidation were about the same as those exhibited for the reduction of OAA but the magnitude of stimulation when compared with that obtained in the reverse reaction was considerably reduced. This reduction in magnitude of stimulation possibly may have been due to the relatively high substrate concentration and the high pH which was necessary to reverse the reaction.

Discussion

In general, the optimum concentration of the different salts required to produce maximum activity of malic dehydrogenase depended to a large extent on the charge of the ions involved. When inorganic salts other than Cl^- and F^- were used, maximum activity was produced by 2–1 electrolytes at approximately the same ionic strength of the medium which resulted in maximum activity with 1–1 electrolytes. Likewise, enzyme stimulation by sodium salts of organic acids also appeared to be related to the ionic strength of the medium. The concentrations of organic salts required for maximum activity, however, were higher than those of the inorganic salts. The reason for this difference is not evident.

The shape of the stimulation curves obtained when enzyme activity was plotted against concentrations of fluoride or chloride salts of univalent cations cannot be explained completely on the basis of the contribution of these salts to the ionic strength of the medium. The results in figure 4 indicate that the first optimum in the NaCl stimulation curve is non-specific and is dependent upon the total salt concentration. The other optima in the curves obtained with Cl^- or F^- appear to be due to a more specific effect of these anions. In general, the enzyme activity increases with increasing ionic strength until an optimum is reached. At concentrations higher than this optimum the general effect of increasing the ionic strength is to inhibit enzyme activity. In the case of Cl^- and F^-, however, apparently there are other effects in addition to the non-specific salt effect.

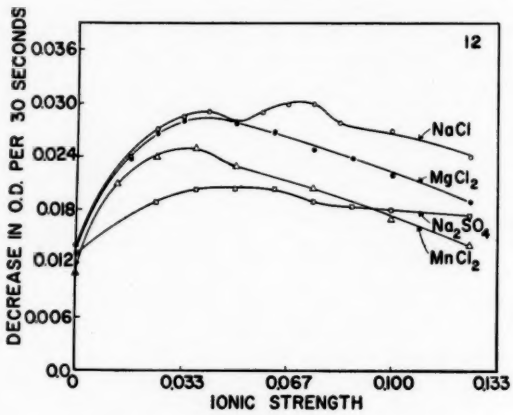
salt divided by the activity in the absence of salt. The enzyme extracts contained approximately six μg of protein. Experiments with the various salts were carried out with separate dilutions of the partially purified extract.

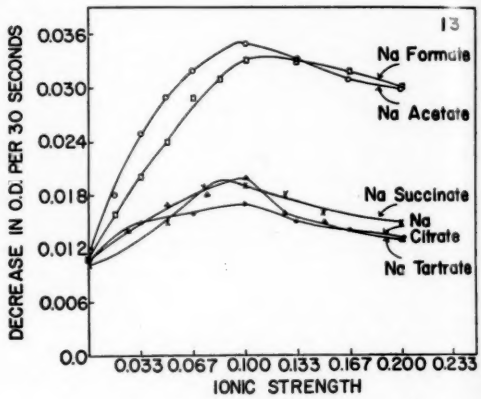
Fig. 9. Effect of NaCl concentration on enzyme activity at fixed levels of $NaNO_3$ concentration. The standard assay procedure was used with variation in concentrations of NaCl and $NaNO_3$ as indicated. The enzyme consisted of approximately seven μg of protein from the partially purified extract.

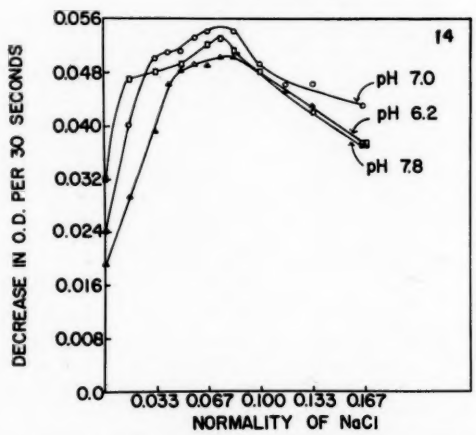
Fig. 10. Reciprocal plots of OAA concentration versus reaction velocity of malic dehydrogenase at different concentrations of NaCl. The standard assay procedure was used with variation in concentrations of OAA and NaCl as indicated. The enzyme consisted of approximately 15 μg of protein from the partially purified extract.

Fig. 11. Reciprocal plots of DPNH concentration versus reaction velocity of malic dehydrogenase at different NaCl concentrations. The standard assay procedure was used with variation in concentrations of DPNH and NaCl as indicated. The enzyme consisted of approximately 15 μg of protein from the partially purified extract.

A possible explanation for the non-specific stimulatory effect of salts on malic dehydrogenase activity may lie in the influence of these salts on the rates of association and dissociation of the substrates and products from the enzyme. Anions have been shown to increase the dissociation of old yellow enzyme into FMN and apoenzyme (22). Also, it is reported that anions influence the association and dissociation velocity constants of DPN and DPNH with alcohol dehydrogenase (23). The influence of salts on the activity of malic dehydrogenase might be explained according to the interpretation by Rutter (20) of the influence of salts on the activity of glucose-6-phosphate dehydrogenase. In any enzymatically catalyzed reaction the catalytic process may be considered to be composed of several partial reactions: A, the association of the enzyme with its substrates, B, the catalytic process, and C, the dissociation of the enzyme from the products of the reaction. The observed overall rate of the reaction must be a function of the rate constant of any one or combination of the partial reactions which are rate limiting. In







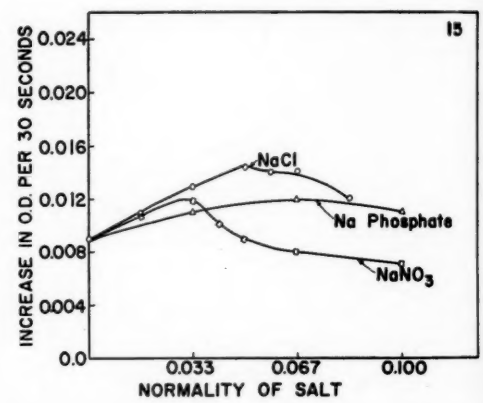


FIG. 12. Effects of various ionic strengths of sodium, magnesium and manganese chlorides and sodium sulfate on malic dehydrogenase activity. The standard assay procedure was used with variation in salt ionic strengths as indicated. The enzyme extracts contained approximately six μg of protein. Experiments with various salts were carried out with separate dilutions of the partially purified extracts.

FIG. 13. Effects of varying ionic strengths of sodium salts of different organic acids on malic dehydrogenase activity. The standard assay procedure was used with variation in the ionic strengths as indicated. The enzyme extract contained approximately six μg of protein. Experiments with the various salts were carried out with separate dilutions of the partially purified extract.

FIG. 14. Effect of NaCl concentration on enzyme activity at different pH values. The reaction mixture was maintained at the indicated pH values by 6.7×10^{-3} M potassium phosphate buffer. The enzyme consisted of approximately 12 μg of the partially purified extract.

FIG. 15. Effect of sodium salts of chloride, phosphate, and nitrate on the reduction of OAA to malate by malic dehydrogenase. The rates of the reactions were assayed as described under Materials and Methods. The enzyme consisted of approximately 50 μg of protein from the partially purified extract.

the case of glucose-6-phosphate dehydrogenase Rutter (20) postulated that at higher ionic strengths the tendency for substrates and products to dissociate from the enzyme was increased. The stimulatory effects of ions on glucose-6-phosphate dehydrogenase activity were explained on this basis by assuming that the dissociation of the products from the enzyme was rate limiting. Adding salts to the malic dehydrogenase reaction resulted in an increase in K_M values of the enzyme with respect to both DPNH and OAA. This indicates that the affinity of the enzyme for these substrates and/or the products is decreased by salts, however, no information is available indicating the portion of the enzymic process which is affected by salts. Since the level of substrate in the assay medium is in excess and therefore is sufficient to saturate the enzyme the effect of an increased rate of dissociation would be overcome by a mass action effect. If the dissociation of the enzymes from the product is the rate-limiting step then the net effect of increasing the rate of dissociation by the addition of salts would be an increase in the observed overall rate of the reaction.

Massey (12) observed that certain anions stimulated salt-free fumarase while other anions inhibited activity. The magnitude of stimulation or inhibition was not the same at different pH levels. He interpreted this phenomenon on the basis that the dissociation of both acidic and basic groups of the enzyme determines the activity and that the pH-activity curve is determined by the dissociation constants of these groups. According to his interpretation the possibility of stimulation or inhibition of enzyme activity depended on whether or not the ions altered the basic dissociation constant to a greater extent than the acidic dissociation constant. The data from figure 14 indicate that the magnitude of stimulation of malic dehydrogenase by added salts is greater in an acid than in an alkaline medium. However, when the contribution of the phosphate buffer to the ionic strength of the medium is taken into account the magnitude of stimulation at the various pH levels is not greatly different if activity is plotted against ionic strength. Therefore, these data do not indicate that the stimulation of activity is due to an influence of salts on the dissociation of acidic or basic groups of the enzyme.

A possible explanation for the occurrence of multiple optima in the Cl^- and F^- stimulation curves is that these anions cause some physical change in the enzyme structure which exposes additional active sites or renders the existing sites more readily available to substrates. Wolfe and Neilands (28) and Davies and Kun (4) have proposed that malic dehydrogenase has more than one site for the substrate. Davies and Kun (4) assumed that the enzyme has one site for the substrate which is catalytically active and another site which forms an enzyme-substrate complex which may inhibit or activate the enzyme depending on the kind of substrate present. It is conceivable that Cl^- or F^- might activate the inactive site. Of interest in this respect is the capacity of F^- and Cl^- to form hydrogen bonds (17). One might postulate that these ions may compete with the intramolecular hydrogen bonding of the enzyme protein resulting in some physical change (e.g., unfolding in the enzyme).

In order to determine whether other dehydrogenase systems in plants are influenced by salts in a manner similar to that found with malic dehydrogenase, some salt studies were conducted on the TPN requiring malic enzyme. In this reaction malate is oxidatively decarboxylated to yield pyruvate. KCl and NaCl failed to stimulate malic enzyme at any concentration, and concentrations of 0.1 *N* decreased the activity by 30 %. The enzyme was inhibited 50 % by either 0.07 *N* $NaNO_3$ or 0.03 *N* $CaCl_2$. These results illustrate that the effect of salts may be very different with different enzyme systems. This is particularly interesting in respect to the possible influence of salt concentrations on metabolic pathways.

It is interesting to note that the concentrations of salts producing maximum stimulation of malic dehydrogenase and the magnitude of increase in the activity of the enzyme resulting from salts are of the same order as those reported for salt respiration by Robertson and Wilkins (10). The possibility exists that this and similar enzyme systems may prove to be involved in the salt stimulated respiratory process in plants.

Summary

Malic dehydrogenase from an acetone powder of spinach leaves was purified 98-fold by procedures involving heat sensitization in the presence of ammonium sulfate, ethanol fractionation, column chromatography, and ammonium sulfate fractionation. Electrophoresis of this preparation on filter paper revealed only two protein bands.

The product of the reduction of oxaloacetate by malic dehydrogenase was identified as L-malate.

The K_M values for oxaloacetate and DPNH were found to be 1.8×10^{-5} M and 1.5×10^{-5} M, respectively.

The optimum pH for malic dehydrogenase activity ranged between 7.0 and 7.4 when sodium phosphate was used as the buffer. The optimum pH value appeared to be influenced by the ionic species that are employed in the buffer system.

Detailed studies have been carried out to determine the influence of different concentrations of salts on the activity of the enzyme. Optimal concentrations of sodium salts of chlorine, fluorine, iodine, bromine, nitrate, and phosphate increased the activity of the enzyme by two- to three-fold. Potassium salts of these anions produced similar effects. Sodium salts of univalent anions produced maximum activity at concentrations of 0.033 to 0.040 M. In addition to this, chloride salts of univalent cations produced a second maximum at a concentration of 0.075 M, while fluorides produced a series of maxima. The occurrence of the first maximum appeared to be due to a non-specific salt effect while the occurrence of maxi-

ma at higher salt concentrations was specific for Cl^- or F^-.

Chloride salts of divalent cations produced a two-fold stimulation and optimal concentrations ranged between 0.12 and 0.013 M. This stimulation seemed to be related to the ionic strength of the medium indicating that the electrical environment of the medium may be an important factor in the phenomenon. Sodium salts of organic acids stimulated enzyme activity maximally at an ionic strength of the medium of 0.1. Possible mechanisms by which salts exert their influence on malic dehydrogenase activity have been discussed.

Literature Cited

1. Anderson, B. 1933. Uber Co-Zymaseaktivierung einiger Dehydrogenasen. Z. physiol. Chem. 217: 186–190.
2. Berger, J. and G. S. Avery, Jr. 1943. Dehydrogenases of Avena coleoptile. Amer. Jour. Bot. 30: 290–297.
3. Bonner, J. and S. T. Wildman. 1946. Enzymatic mechanisms in the respiration of spinach leaves. Arch. Biochem 10: 497–518.
4. Davies, D. D. and E. Kun. 1957. Isolation and properties of malic dehydrogenase from ox-heart mitochondria. Biochem. Jour. 66: 307–316.
5. Eichel, H. J. 1956. Respiratory enzyme studies in *Tetrahymena pyriformis*. III. Some properties of reduced diphosphopyridine nucleotide oxidase of cell-free homogenates. Jour. Biol. Chem. 222: 137–144.
6. Honda, S. E., R. N. Robertson, and Jeanette M. Gregory. 1958. Studies in the metabolism of plant cells. XII. Ionic effects on oxidation of reduced diphosphopyridine nucleotide and cytochrome c by plant mitochondria. Australian Jour. Biol. Sci. 11: 1–15.
7. Horecker, B. L. and A. Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. Jour. Biol. Chem. 175: 385–390.
8. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666.
9. Lowry, D. H., Nira J. Rosebrough, A. L. Farr, and Rose J. Randall. 1951. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275.
10. Lundegardh, H. 1955. Mechanism of absorption, transport, accumulation, and secretion of ions. Ann. Rev. Plant Physiol. 6: 1–24.
11. Lynen, F. and W. Franke. 1941. Zur Kenntnis der Co-Enzymspezifitat von Dehydrasen. I. Malico-und Glucose-dehydrase. Z. physiol. Chem. 270: 271–280.
12. Massey, V. 1953. Studies on fumarase. II. The effect of inorganic ions on fumarase activity. Biochem. Jour. 53: 67–71.
13. Miller, G. W. and H. J. Evans. 1956. The influence of salts on the activity of particulate cytochrome oxidase from roots of higher plants. Plant Physiol. 31: 357–364.
14. Ochoa, S. 1955. Malic dehydrogenase from pig heart. In: Methods of Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. I: Pp. 735–739. Academic Press Inc., New York.
15. Ochoa, S. 1955. Malic enzyme. In: Methods of Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. I: 739–741 Academic Press Inc., New York.
16. Palmer, J. K. 1955. Chemical investigations of the tobacco plant. X. Determination of organic acids by ion exchange chromatography. Bull. 589. Conn. Agr. Expt. Station, New Haven.
17. Pauling, L. 1948. The Nature of the Chemical Bond. 2nd edition, Cornell University Press, Ithaca.
18. Robertson, R. N. 1951. Mechanism of absorption and transport of inorganic nutrients in plants. Ann. Rev. Plant Physiol. 2: 1–24.
19. Robertson, R. N. and Marjorie J. Wilkins. 1948. Studies in the metabolism of plant cells. VIII. The quantitative relation between salt respiration. Australian Jour. Sci. Research Ser. B 1: 17–37.
20. Rutter, W. J. 1957. The effect of ions on the catalytic activity of enzymes: yeast glucose-6-phosphate dehydrogenase. Acta Chem. Scand. 11: 1576–1586.
21. Straub, F. B. 1942. Reinigung der Apfelsauredehydrase und die Bedeutung der Zellstruktur in der Apfelsauredehydrierung. Z. physiol. Chem. 275: 63–72.
22. Theorell, H. and A. P. Nygaard. 1954. The combination of flavine mononucleotide and riboflavin with the protein of "the old yellow enzyme." Acta Chem. Scand. 8: 1104–1105.
23. Theorell, H., A. P. Nygaard, and R. Bonnichsen. 1955. Studies on liver alcohol dehydrogenase. III. The influence of pH and some anions on the reaction velocity constants. Acta Chem. Scand. 9: 1148–1165.
24. Thunberg, T. 1929. Uber das Vorkommen einer Citrico-Dehydrogenase in Gurkensamen und ihre Verwertung fur eine hochempfindliche biologische Farbenreaktion auf Zitronensaure. Biochem. Z. 206: 109–119.
25. Vestling, C. S., T. Huang, F. C. Grimm, A. C. Boyer, A. K. Khettry, and R. H. Hook. 1959. Rat liver malic dehydrogenase purification. Federation Proc. 18: 343.
26. Wagner-Jauregg, T. and H. Rauen. 1935. Uber die enzymatische Dehydrierung der Zitronensaure. Z. physiol. Chem. 233: 215–222.
27. Walaas, E. and O. Walaas. 1956. Kinetics and equilibria in flavoprotein systems. V. Effects of pH, anions, and partial structural analogues of coenzyme on activity of D-amino acid oxidase. Acta Chem. Scand. 10: 122–133.
28. Wolfe, R. G. and J. B. Neilands. 1956. Some molecular and kinetic properties of heart malic dehydrogenase. Jour. Biol. Chem. 221: 61–69.
29. Zelitch, I. 1955. The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. Jour. Biol. Chem. 216: 553–575.

INFLUENCE OF CERTAIN CATIONS ON ACTIVITY OF ACETIC THIOKINASE FROM SPINACH LEAVES[1,2]

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The first evidence for a cofactor necessary in acetate metabolism was provided by Nachmansohn and Machado (13) who discovered a soluble enzyme system in extracts of brain tissue which catalyzed the acetylation of choline in the presence of adenosine triphosphate (ATP). Lipmann (8) and Kaplan and Lipmann (5) described an enzyme system in extracts of pigeon liver which catalyzed the ATP and coenzyme A (CoA) dependent acetylation of sulfanilamide. These investigators were the first to identify CoA as a necessary factor in acetate activation. Final proof of the constitution of active acetate was provided when Lynen and Reichert (10) isolated and identified acetyl CoA.

Enzyme extracts were obtained from pig heart and rabbit heart by Beinert et al (1) and pigeon liver and yeast by Jones et al (4) which catalyzed the reversible reaction of ATP, acetate, and CoA to yield acetyl CoA, adenosine monophosphate (AMP), and inorganic pyrophosphate (PP). The overall reaction is formulated in the following equation:

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{AMP} + \text{PP}$$

Acetic thiokinase was extracted from the tissues of higher plants by Millerd and Bonner (12). The enzyme was obtained by these authors from every tissue in which it was sought; however, the most active preparations were obtained from spinach leaves. The enzyme appeared to be associated with the mitochondria in both plant and animal organisms.

Acetic thiokinase has been shown to require Mg^{++} (1, 4, 12) for activity. In addition Jones et al (4) reported that the reaction rate appeared to be increased in a medium containing K^+. Von Korff (15) made a study of the effects of alkali metals on the overall acetate activation system from animal tissues and concluded that the reaction was absolutely dependent upon univalent cations and that maximum activity was obtained with K^+, Rb^+, or NH_4^+ at concentrations of 0.04 M. Na^+ or Li^+ strongly inhibited the reaction. The inhibition by Na^+, however, was not overcome by K^+ indicating that the inhibition was not due to a competition between Na^+ and K^+. The alkali metal requirements of acetic thiokinase from higher plants has not been reported in the literature.

Berg (2, 3) has demonstrated that the reaction catalyzed by acetic thiokinase proceeds in two steps. These are formulated in the following equations:

$$\text{ATP} + \text{Acetate} \rightleftharpoons \text{Adenyl acetate} + \text{PP}$$

$$\text{Adenyl acetate} + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{AMP}$$

All attempts to demonstrate the involvement of more than one enzyme in the two reactions have been negative (3). According to Berg (3) Mg^{++} is required for the first step of the above sequence. This was demonstrated by measuring ATP formation in the presence of adenyl acetate, PP, and acetic thiokinase. Maximum activity was produced with Mg^{++} at a concentration of 1.5×10^{-3} M. In contrast to the Mg^{++} requirement for the conversion of adenyl acetate to ATP, the reaction of adenyl acetate and CoA yielding acetyl CoA was maximal in the absence of Mg^{++}. No study has been conducted to determine the possible univalent cation requirements of the individual reactions in the acetate activation sequence.

In view of the importance of acetate in plant metabolism and of the limited information concerning the role of univalent cations in plant metabolism it was considered that a detailed study of the requirements of univalent cations for acetate activation in plants was appropriate.

MATERIALS AND METHODS

PREPARATION OF ACETIC THIOKINASE: Acetic thiokinase was obtained from an acetone powder of the leaves of spinach (*Spinacea oleracea* L.). The acetone powder was prepared according to the procedure described by Millerd and Bonner (12). All reagents used and all operations involved in the preparation of the enzyme were maintained between 0 and 4° C. In preparing the crude extract the acetone powder was ground with mortar and pestle for 5 minutes with 20 weights of 0.02 M tris (hydroxymethyl)aminomethane (Tris) buffer at pH 7.4. The suspension was centrifuged for 10 minutes at a force of 15,000 × G and the precipitate discarded. The concentration of nucleic acids in the supernatant solution was determined spectrophotometrically by the procedure described by Layne (6) and 0.025 ml of 2% protamine sulfate was added per milligram of nucleic acid in the extract. The suspension was centrifuged for 10 minutes at 15,000 × G and the precipitate was discarded. To the supernatant solution was added with stirring, sufficient solid ammonium sulfate to bring the solution to 30% of saturation. After 10 minutes the precipitate was removed by centrifugation and the supernatant was brought to 50% of saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a third the original volume of 0.02 M Tris

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buffer at pH 7.4. This extract contained a small amount of reduced diphosphopyridine nucleotide (DPNH) oxidase which was essentially removed by refractionation with ammonium sulfate. In this procedure the extract was brought to 33 % of saturation with ammonium sulfate and the precipitate was collected by centrifugation and discarded. Sufficient ammonium sulfate was added to the supernatant liquid to bring the concentration to 50 % of saturation. The precipitate was collected by centrifugation and redissolved in a sixth the original volume of 0.02 M Tris buffer, pH 7.4. This procedure produced a two-fold purification of the enzyme. The amount of DPNH oxidase activity present in this extract was negligible and therefore did not interfere with the acetic thiokinase assays. The extract was dialyzed for 3 hours against 0.001 M Tris buffer at pH 8.0. After this period the dialyzing solution was renewed and dialysis was continued for 3 additional hours. Extracts prepared by this procedure were used for the various experiments.

PREPARATION OF CONDENSING ENZYME: Partially purified condensing enzyme was prepared from pig hearts by a procedure modified from the method outlined by Ochoa (14). Two pig hearts were chilled in ice as rapidly as possible after removal from the animal. The fat and connective tissue were removed and the muscle tissue was ground in a food chopper. The resulting mince was stirred for 30 minutes with 0.02 M Tris buffer, pH 7.4 (2 ml/g of wet mince). The suspension was squeezed through four thicknesses of cheesecloth and the residue was re-extracted with Tris buffer at pH 7.4 as described above. The two extracts were combined and adjusted to pH 5.5 by slowly adding 0.5 M acetic acid with mechanical stirring. Calcium phosphate gel was then added in the proportion of 0.3 mg gel solids per milligram of protein. The suspension was stirred for 4 hours and allowed to settle overnight. The clear red supernatant solution was siphoned off, and the gel was collected by centrifugation. The supernatant fluid was discarded and the condensing enzyme was extracted from the gel by shaking it for 30 minutes with 50 ml of 0.1 M potassium phosphate buffer at pH 7.4. Several small glass beads were added to aid in breaking up the precipitate. After shaking, the suspension was centrifuged and the supernatant fluid was collected. The gel was re-extracted six times and the eluates were combined.

Further purification was accomplished by ammonium sulfate fractionation. To the combined eluates was added slowly, with stirring, sufficient solid ammonium sulfate to bring the solution to 40 % of saturation. The precipitate was removed by centrifugation and the supernatant solution was brought to 70 % of saturation with ammonium sulfate. The precipitate was collected by centrifugation and redissolved in a tenth the original volume of 0.02 M Tris buffer at pH 7.4. All operations were carried out at 0 to 4° C.

This procedure resulted in a ten-fold purification. The purified extract was free of acetic thiokinase and DPNH oxidase activity. Before use in acetic thiokinase assays the extract was dialyzed 12 hours against 0.001 M Tris buffer at pH 7.4.

OTHER MATERIALS: Diphosphopyridine nucleotide (DPN) of 95 to 100 % purity and ATP of 99 to 100 % purity were obtained from the Sigma Chemical Co., St. Louis, Mo. CoA of 75 % purity, malic acid, and glutathione were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Other chemicals were of reagent grade and were obtained from commercial sources. The Tris salts of DPN and ATP were obtained by passing solutions and the sodium salts of these coenzymes through an exchange column containing Dowex 50 resin (Tris cycle). The Tris salts of acetate and malate were prepared by neutralizing the respective acids to pH 8.0 with Tris. Acetyl CoA was prepared from acetic anhydride and CoA according to the procedure outlined by Ochoa (14).

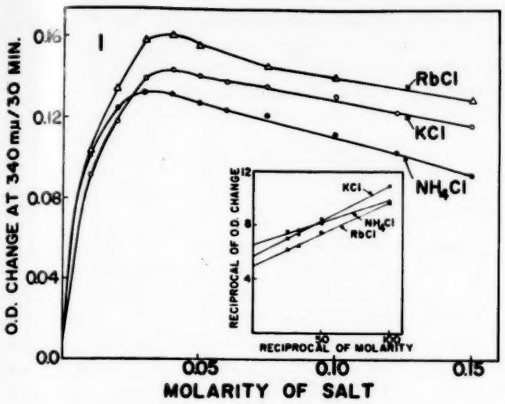
STANDARD ASSAY PROCEDURES: Acetic thiokinase was assayed by a method essentially the same as that described by Millerd and Bonner (12). Activity was followed spectrophotometrically by measuring the rate of reduction of DPN at a wave length of 340 mμ in a system involving the coupling of malic dehydrogenase, acetic thiokinase, and condensing enzyme. These reactions, in the proper sequence are as follows:

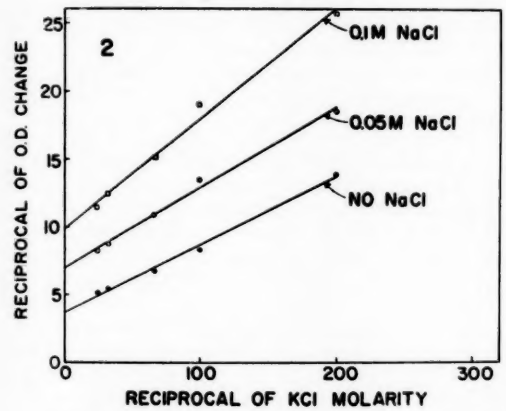
$$\text{Malate} + \text{DPN} \rightleftharpoons \text{Oxaloacetate} + \text{DPNH} + \text{H}^+$$

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{AMP} + \text{PP}$$

$$\text{Oxaloacetate} + \text{Acetyl CoA} \rightleftharpoons \text{Citrate} + \text{CoA}$$

In this assay the rate of reduction of DPN is equal to the rate of citrate synthesis (14). The standard reaction mixture in a final volume of 1 ml contained the following constituents: 100 micromoles of Tris buffer adjusted to pH 8.0 with HCl; 10 micromoles of Tris acetate; 2.5 micromoles of $MgCl_2$; 50 micromoles of KCl; 5 micromoles of Tris malate; 10 micromoles of glutathione; 5 micromoles of Tris ATP; 0.67 micromole Tris DPN; 0.09 micromole of CoA; 20 to 30 units of condensing enzyme and acetic thiokinase extract usually containing between 0.6 and 1.0 mg of protein. The quantities, in micromoles, of ATP, CoA, acetate, and DPN required for maximum enzyme activity in a 1 ml reaction mixture were 2.5, 0.09, 5.0, and 0.4, respectively. Both the condensing enzyme extract and the acetic thiokinase extract contained large amounts of malic dehydrogenase. Sufficient malic dehydrogenase and condensing enzyme were present in the reaction mixture, therefore, to insure that acetic thiokinase was the rate-limiting step. The mixtures were incubated 30 minutes at 30° C and the reaction was stopped by adding 5 ml of 0.5 M sodium phosphate buffer at pH 7.2. DPNH formed under the desired experimental conditions was compared with a control reaction containing all the components except CoA. The reaction rate was proportional to acetic thiokinase concentration up to a con-





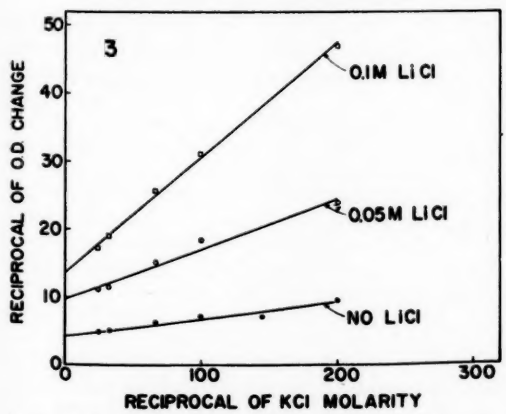


FIG. 1 (*major graph*). The influence of various concentrations of univalent cations on the activity of acetic thiokinase. The standard assay procedure was used with variation in concentration of univalent cations as indicated. The dialyzed enzyme extract added to each reaction mixture contained 0.7 mg. of protein.

(*Inset*) Reciprocal plots of reaction velocities versus salt concentrations indicated in the major graph. Only those points were utilized in the major graph that were

TABLE I

ESSENTIALITY OF VARIOUS COMPONENTS OF STANDARD ASSAY MEDIUM FOR ACETIC THIOKINASE*

REACTION MIXTURE	ACTIVITY RELATIVE TO COMPLETE MIXTURE
Complete	100
No ATP	0
No CoA	0
No Acetate	15
No $MgCl_2$	0
No KCl	0
No DPN	0
No malate	0
No condensing enzyme	0

* The complete reaction mixture in a volume of 1 ml contained the following constituents in micromoles: Tris buffer, pH 8.0, 100; Tris acetate, 10; $MgCl_2$, 2.5; Glutathione, 10; Tris ATP, 5; Tris DPN, 0.67; CoA, 0.09; KCl, 50: Tris malate, 5; condensing enzyme (20–30 units) and acetic thiokinase (1.0 mg protein.).

centration of 2.0 mg of protein in each assay vessel. No activity was obtained when the active enzyme was replaced in the standard mixture by a boiled enzyme preparation. Evidence for the dependence of enzyme activity on the various constituents of the assay medium is presented in table I.

In addition to the procedure involving coupling of the acetic thiokinase reaction with the condensing enzyme and malic dehydrogenase reactions, incubation mixtures containing hydroxylamine prepared as described by Millerd and Bonner (12) exhibited positive acetate dependent hydroxamic acid reactions.

Condensing enzyme was assayed according to the optical procedure outlined by Ochoa (14). One unit

free of apparent inhibition. Calculation of regression lines by the method of least squares gave the following regression equations: K^+, $Y = 5.65 + 0.054\ X$, NH_4^+, $Y = 6.56 + 0.033\ X$; Rb^+, $Y = 4.98 + 0.048\ X$.

FIG. 2. Reciprocal plots of reaction velocities versus KCl concentration at different NaCl concentrations. The standard assay procedure was used with variation in salts as indicated. The dialyzed enzyme extract added to each reaction mixture contained 1.0 mg of protein. Calculation of regression lines by the method of least squares gave the following regression equations: No NaCl, $Y = 3.69 + 0.050\ X$; 0.05 M NaCl, $Y = 6.98 + 0.059\ X$; 0.1 M NaCl, $Y = 9.77 + 0.082\ X$.

FIG. 3. Reciprocal plots of reaction velocities versus KCl concentration at different levels of LiCl concentration. The dialyzed enzyme extract added to each reaction mixture contained 1.0 mg of protein. The standard assay procedure was used with variation in salt as indicated. Calculation of regression lines by the method of least squares gave the following regression equations: No LiCl, $Y = 4.27 + 0.26\ X$; 0.05 M LiCl, $Y = 9.6 + 0.074\ X$; 0.1 M LiCl, $Y = 13.45 + 0.17\ X$.

of condensing enzyme is defined as that amount which gives an optical density change of 0.01 per minute under the standard assay conditions. Protein was determined by the Folin-phenol method of Lowry et al (9) using bovine albumin as the standard.

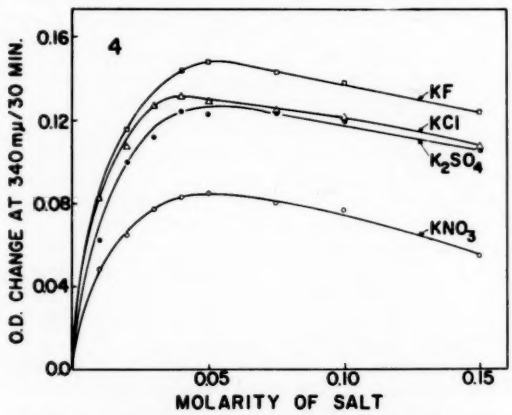
Results

Univalent Cation Activation: The curves in figure 1 (major graph) illustrate the influence of chloride salts of K^+, NH_4^+, and Rb^+ on the activity of acetic thiokinase. In these experiments an extract was utilized that had been dialyzed for 6 hours against 0.001 M Tris buffer at pH 8.0. As indicated by these curves the enzyme activity was absolutely dependent upon univalent cations. Periods of dialysis less than 6 hours were not sufficient to remove all endogenous univalent cations. Maximum enzyme activity was exhibited with chloride salts of K^+, NH_4^+, or Rb^+ at a concentration of 0.04 M. The addition of these salts at levels higher than 0.04 M resulted in inhibition. Using the points on the curve which were free of apparent inhibition, plots were constructed (inset graph, fig 1) according to the method of Lineweaver and Burk (7) and the Michaelis constants (K_A) for the activating cations were calculated. K_A values for K^+, NH_4^+, and Rb^+ were, respectively: 9×10^{-3} M; 5×10^{-3} M, and 9×10^{-3} M. These values are only approximations, however, because concentrations of salts greater than 0.04 M resulted in inhibition. This was most apparent with NH_4Cl.

The enzyme was not activated by Na^+ or Li^+ and inhibition was produced by low concentrations of these cations. When a concentration of 0.05 M Na^+ or Li^+ was included in the standard assay mixture, enzyme activity with the two ions was inhibited 38 % and 56 %, respectively. To determine whether or not the inhibition produced by Na^+ or Li^+ was due to competition of these cations with the activating univalent cations, KCl saturation curves were determined at various concentrations of Na^+ or Li^+. Lineweaver-Burk plots of these data are presented in figures 2 and 3. Failure of the projected curves to intersect at infinite KCl concentration demonstrates that the inhibition by Na^+ or Li^+ is not competitive with respect to K^+.

Effect of Various Anions of Enzyme Activity: As indicated by the curves in figure 4, the influence of univalent cation salts on enzyme activity was not independent of the balancing anion. Although the optimum concentration varied slightly with potassium salts of different anions, maximum enzyme activity varied markedly with different potassium salts. Enzyme activity in the presence of the nitrate salt of potassium was 55 % of the activity in the presence of chloride. The activity obtained with potassium sulfate was intermediate between the activities obtained with potassium fluoride and potassium nitrate.

Divalent Cation Activation of Acetic Thiokinase: In addition to the requirement for univalent cations for activity, acetic thiokinase from spinach exhibits an absolute requirement for divalent cations. The effect of various divalent cation chlorides on the enzyme activity is illustrated by the curves in figure 5. In these experiments the standard assay procedure was used with the exception that divalent cation salts were included in the assay mixture as indicated. The activating capacities of Mg^{++} and Mn^{++} were approximately equal at optimal concentrations. Ca^{++} was 77% as effective as Mg^{++} or Mn^{++}, and Ba^{++} failed to activate the enzyme at any concentration tested. Maximum enzyme activity was observed at



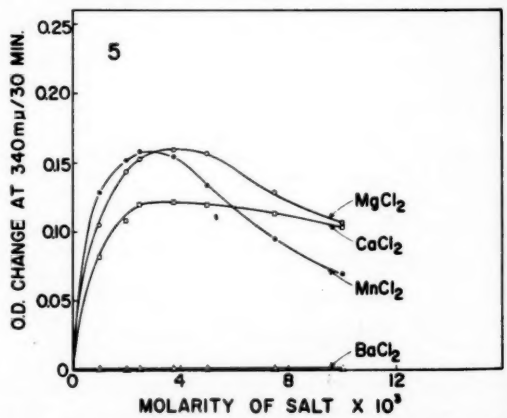


Fig. 4. The influence of various concentrations of potassium salts of several anions on the activity of acetic thiokinase. The standard assay procedure was used with variation in anions as indicated. The dialyzed enzyme extract added to the reaction mixture contained 0.7 mg of protein.

Fig. 5. The influence of various divalent cations on the activity of acetic thiokinase. The standard assay procedure was used with variation in divalent cations as indicated. The dialyzed enzyme extract added to the reaction mixture contained 0.8 mg of protein.

a Mg^{++}, Mn^{++}, or Ca^{++} concentration of approximately 3×10^{-3} M. Activity was markedly inhibited at concentrations higher than optimal.

Discussion

Acetic thiokinase from spinach exhibits an absolute requirement for univalent cations. Maximum enzyme activity was produced by K^+, NH_4^+, or Rb^+ at a concentration of 0.04 M. A similar concentration of these cations produced maximum activity of the enzyme from animal tissues (15). Enzyme activity was inhibited by Na^+ or Li^+ and the inhibition was not competitive with respect to the activating univalent cations. The influence of these ions on acetic thiokinase from animals (15) was similar to that observed for the plant enzyme.

The failure of Na^+ to replace K^+ in the activation of acetic thiokinase is in contrast with the capacity of this cation to function as an activator for pyruvate kinase from higher plants (11). One might postulate that the acetic thiokinase reaction may represent an important site of physiological antagonism between K^+ and Na^+ in higher plant metabolism.

The reason for the differences in maximum enzyme activity obtained when different anions were used in conjunction with a common cation is not readily apparent. Perhaps there is a total salt or anionic inhibition which partially overcomes the activating effect of the univalent cation. Further investigation is necessary to clarify this observation.

In addition to its requirement for univalent cations the activity of acetic thiokinase also is dependent upon divalent cations. This requirement was satisfied by Mg^{++}, Mn^{++}, or Ca^{++}; however, the maximum activity with the latter cation was only 77 % of that with either Mg^{++} or Mn^{++}. Further experimentation would be necessary in order to postulate reasons for the variability in activating capacities of different divalent cations.

In regard to the physiological importance of various univalent cation activators of this system it is concluded that the concentrations of either NH_4^+ or Rb^+ normally present in fresh plant material are insufficient to play an important role in activating this enzyme. In view of this conclusion and of the toxicity of high concentrations of either NH_4^+ or Rb^+ to plant tissues, the most probable physiologically active univalent cation for this enzyme is K^+. In this regard fresh plant material normally contains about 0.2 % of K^+. This concentration is very near to the 0.04 M concentration required for maximum activity of the enzyme in vitro.

Experiments are presently being conducted to determine the possible role of univalent cations in the individual reactions of the acetate activation sequence. Preliminary experiments have indicated that the conversion of adenyl acetate and CoA to acetyl CoA and AMP is stimulated by potassium ions.

Summary

Acetic thiokinase was obtained from an acetone powder of the leaves of Spinach (*Spinacea oleracea* L.). Dialyzed extracts of this enzyme required both univalent and divalent cations for activity. The univalent cation requirement was satisfied by K^+, NH_4^+, or Rb^+ and maximum activity was obtained in the presence of 0.04 M concentration of these cations. Enzyme activity was inhibited when Na^+ or Li^+ was added to the standard assay mixture. This inhibition apparently was not due to a competition of these cations with the activating univalent cations.

The divalent cation requirement for enzyme activity was satisfied by Mg^{++}, Mn^{++}, or Ca^{++}. Ba^{++} was ineffective at all concentrations tested.

In general it is concluded that the cation requirements for acetic thiokinase from spinach leaves are very similar to those that have been established for this enzyme from animal sources.

Literature Cited

1. Beinert, H., D. E. Green, P. Hele, Helen Hift, R. W. Von Korff, and C. V. Ramakrishnan. 1953. The acetate activating enzyme system of heart muscle. Jour. Biol. Chem. 203: 35–45.
2. Berg, P. 1955. Participation of adenyl-acetate in the acetate-activating system. Jour. Amer. Chem. Soc. 77: 3163–3164.
3. Berg, P. 1956. Acyl adenylates: an enzymatic mechanism of acetate activation. Jour. Biol. Chem. 222: 991–1013.
4. Jones, Mary Ellen, S. Black, Ruth M. Flynn, and F. Lipmann. 1953. Acetyl CoA synthesis through pyrophosphoryl split of adenosine triphosphate. Biochem. Biophys. Acta 12: 141–149.
5. Kaplan, N. O. and F. Lipmann. 1948. The assay and distribution of coenzyme A. Jour. Biol. Chem. 174: 37–44.
6. Layne, E. 1955. Spectrophotometric and turbidometric methods for measuring protein. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. III. Pp. 447–454. Academic Press Inc., New York.
7. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 36: 658–666.
8. Lipmann, F. 1945. Acetylation of sulfanilimide by liver homogenates and extracts. Jour. Biol. Chem. 160: 173–190.
9. Lowry, D. H., Nira J. Rosebrough, A. L. Farr, and Rose J. Randall. 1951. Protein measurement with the Folin phenol reagent. Jour. Biol. Chem. 193: 265–275.
10. Lynen, F. and E. Reichert. 1951. Zur Chemischen struktur activierten Essigsaure. Angew. Chem. 69: 47–48.
11. Miller, G. W. and H. J. Evans. 1957. The influence of salts on pyruvate kinase from tissues of higher plants. Plant Physiol. 32: 346–354.
12. Millerd, A. and J. Bonner. 1954. Acetate activation and acetoacetate formation in plant systems. Arch. Biochem. Biophys. 49: 343–355.
13. Nachmansohn, D. and A. L. Machado. 1943. The formation of acetyl choline. A new enzyme: "choline acetylase." Jour. Neurophysiol. 6: 397–403.
14. Ochoa, S. 1955. Crystalline condensing enzyme from pig heart. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. I. Pp. 685–694. Academic Press, Inc., New York.
15. Von Korff, R. W. 1953. The effects of alkali metal ions on the acetate activating enzyme system. Jour. Biol. Chem. 203: 265–271.

A CAROTENE PRECURSOR: ITS PROPOSED STRUCTURE AND PLACE IN BIOSYNTHETIC SEQUENCE [1, 2]

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The isoprenoids, cholesterol and rubber, are known to arise from mevalonic (3,5-dihydroxy-3-methylvaleric) acid (5, 7). The same is true of carotenes (1, 6). Thus it has been found in this laboratory that mevalonic acid is rapidly incorporated into carotenes and related compounds in the tomato fruit (6). The hydrocarbon fraction of tomatoes previously incubated with 2-C^{14}-mevalonate contains radioactivity, however, not only in the known carotenes but also in a previously undescribed, colorless polyene which possesses absorption maxima at 208 mμ and 231 mμ. The high specific activity of this compound, which has been referred to as fraction II (6), suggests that it may be a precursor of carotenes. This paper reports further experiments pertaining to the identity of fraction II and to its role in the biosynthesis of carotenoids.

MATERIALS AND METHODS

SUBSTRATES: Samples of 2-C^{14}-mevalonic acid were obtained from Isotopes Specialties Co., Inc.[1] (Lot #A39111307, 0.92 mc/mM) and from Tracerlab, Inc.[5] (Lot 471-57-20, 1.1 mc/mM) as the N,N′-dibenzylethylene-diamine salt.

RADIOACTIVE ASSAY: C^{14}-containing samples were assayed for radioactivity with a Nuclear-Chicago Corp. Model D47 Micromil gas flow counter.[5] The small amounts of material used made correction for self-absorption unnecessary.

SUBSTRATE ADMINISTRATION: Fruits of commercial tomato varieties were supplied with substrate by injection into the locules by syringe or by vacuum infiltration through the stem scar.

CAROTENE EXTRACTION AND SEPARATION: The tomatoes, after incubation, were ground in methanol, and the carotene-containing hexane extract prepared by the method of Purcell et al (6), with the alteration that the xanthophylls were removed with 95 % methanol rather than upon a silica-methanol column. The hexane extract was chromatographed on 1.8 cm. by 25 cm. columns of magnesium oxide (Fisher Sea Sorb)[5] and Hyflo SuperCel (1 : 1, w : w). The various polyenes were eluted with progressively more polar solvent mixtures, beginning with hexane and ending with 2 % methanol : 10 % acetone : 88 % hexane. Carotenes were eluted from the column in the order of increasing unsaturation, the most highly unsaturated, lycopene, being discharged last. The separation of fractions Ia, Ib, IIa, and IIb from the crude phytoene mixture by chromatography on activated alumina has previously been described (6).

ULTRAVIOLET AND INFRARED SPECTRA: Ultraviolet spectra were obtained on hexane solutions with the Cary Model 11MS Recording spectrophotometer[5]. Infrared spectra were taken with the Perkin-Elmer 137 Infracord Spectrophotometer[5] using sodium chloride cells and chloroform as the solvent.

DEGRADATION PROCEDURES: Ozonolysis of fraction II was carried out by bubbling a stream of oxygen containing approximately 1 % ozone through the sample which was dissolved in 10 ml of chloroform at 0° C. The effluent gas was then led through a trap filled with an aqueous solution of potassium iodide and boric acid. When the sample no longer absorbed ozone, as indicated by the appearance of free iodine in the trap, 0.1 ml of acetic acid was added and ozonization continued for a further 3 minutes. The ozonides were next shaken with 0.2 ml of 30 % H_2O_2 for 15 minutes. This was followed by shaking with a further 0.15 ml of H_2O_2 for 15 additional minutes. Finally, 1.0 ml of H_2O_2 and 1.5 ml of water were added, and the mixture refluxed for 7 hours. Washed air was then bubbled through the mixture and into a solution of 2,4-dinitrophenylhydrazine reagent to trap any volatile carbonyl compounds that might be present. The remaining ozonolysis mixture was acidified with HCl and extracted with ether. Both the ether extract and the aqueous residue were examined by paper chromatography.

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[5] Mention of trade products and brands does not imply that they are endorsed or recommended by the U.S. Department of Agriculture or the California Institute of Technology over similar products and brands not mentioned.

TABLE I

POLYENES PRESENT IN TOMATOES AT DIFFERENT STAGES OF RIPENESS*

FRACTION	STAGE			
	MATURE GREEN	LIGHT PINK**	DARK PINK	TABLE RIPE
Fraction Ia	...	16.6	5.9	5.7
Fraction Ib			5.7	11.8
Fraction IIa	0.345	0.24	0.075	0.029
Fraction IIb	0.293		0.078	...
Phytoene	0	0.067	0.162	1.36
Phytofluene	0	0.005	0.135	0.54
Neo-β-carotene	0.0038***	0.005	0.0043	0.0018
β-Carotene	0.14	0.43	0.35	0.323
ζ-Carotene	0		0.061	0.027
γ-Carotene	0	0.018	0.094	0.025
Lycopene	0	0.085	1.34	6.89

* Quantities expressed in mg/100 g fresh weight.
** This stage is known commercially as the breaker stage.
*** May be mostly α-carotene.

RESULTS

KINETICS OF CAROTENE LABELING: The data previously reported have shown, as pointed out above, that when 2-C^{14}-mevalonic acid or 1-C^{14}-acetic acid are administered to ripening tomatoes, substantial amounts of radioactivity are found in a previously unreported compound, fraction IIa (6). Further experiments have been carried out to test the possibility that fraction IIa may be a carotene precursor. Thus table I concerns experiments in which 2-C^{14} mevalonic acid was supplied to tomato fruit at varying stages of fruit development. The data show that although many of the known carotenes increase from negligible amounts at the time of first color formation by the fruit to rather large amounts at full ripeness, fraction IIa decreases in quantity during this period. Immature green tomatoes contain even more fraction IIa, by a factor of three to four, than fruit at the beginning of color formation. The incorporation of 2-C^{14}-mevalonic acid or of 1-C^{14}-acetate into fraction IIa by such immature fruit is greater than that by mature fruit. Thus at the unripe stage, fraction IIa is being synthesized by the fruit at a rapid rate.

All attempts to bring about a direct conversion of radioactive fraction IIa to carotenes by injecting the former into ripening fruit have met with failure. This may very well be due to the almost complete insolubility of fraction II in aqueous media and to associated permeability difficulties. A different type of experiment has, therefore, been designed to test the fate of fraction IIa. The experiment is based upon the fact that the compound is rapidly synthesized in green fruit. It must first be noted that the incorporation of injected 2-C^{14}-mevalonic acid into non-saponifiable matter is essentially completed within 24 hours (table II). The labeled substrate which remains in the fruit is unavailable to metabolism, possibly due to its accumulation into vacuoles. Experiments in which fruits are injected a single time with mevalonate are therefore *pulse* experiments in which the transfer of label from initial product to successive metabolites of this product may be followed with time. Immature fruit were therefore fed 2-C^{14}-mevalonate and allowed to remain on the plant until color development had begun. Table III compares the distribution of activity among the carotene fractions of tomatoes injected with labeled mevalonate while immature and then incubated for various times either on or excised from the plant. Fruit incubated on the plant show somewhat less total incorporation of mevalonic acid into carotenes due to translocation of label to other parts of the plant.

The radioactivity of 2-C^{14}-mevalonate, after injection into the green fruit, is rapidly incorporated into fraction II of the crude phytoene fraction. Within 6 days after injection, however, the bulk of radioactivity has disappeared from this fraction and has appeared in a fraction which may be eluted from the magnesium oxide-SuperCel column immediately before β-carotene. This fraction remains highly labeled until the commencement of ripening. At this time it loses radioactivity which appears instead in the carotenes proper.

NATURE OF LABELED METABOLITES: Characterization studies have been carried out on the compounds isolated from the crude phytoene fraction of ripening

TABLE II

INCORPORATION OF 2-C^{14}-MEVALONATE INTO NON-SAPONIFIABLE FRACTION OF RIPENING TOMATOES INCUBATED FOR VARIOUS LENGTHS OF TIME

FRUIT WT	SUBSTRATE INJECTED	INCUBATION TIME	INCORPORATION	INCORPORATION/ 100 G FRESH WT
G	CPM	HR	CPM	%
177	1×10^6	4	52,000	2.9
250	1×10^6	24	159,000	6.4
290	1×10^6	48	165,400	5.7
200	1.8×10^6	170	183,000	5.1

tomatoes. Fractions Ia and Ib were grouped together for study because of their similarity in physical characteristics and because of the difficulty in securing a clean chromatographic separation of one from the other. Although it is present in large quantity in both immature and ripe tomatoes, fraction I does not become labeled in fruit injected with 2-C^{14}-mevalonic acid. The fact that 1-C^{14}-acetate is actively incorporated into fraction I indicates that this material may be a straight chain hydrocarbon derived from a fatty acid. In agreement with this view is the fact that ozonolysis of purified fraction I causes no detectable degradation. The molecule is, therefore completely saturated. Molecular weight, as determined by the Rast method (Mr. G. Swinehart) is 327, corresponding to a C_{23} or C_{24} compound. Carbon-hydrogen analysis (Dr. A. Elek) yielded the following duplicate results: C = 82.02 %, H = 12.96 %; C = 81.90 %, H = 13.05 %. The 5 % unaccounted for is sufficiently large to allow for an oxygen atom, but there is no indication from infrared spectra or from chromatographic behavior that an oxygen-containing group is present.

Much of the radioactivity of the phytofluene and neo-β-carotene fractions is due to the presence of an unidentified compound. This colorless substance has no absorption maxima above 208 mμ, melts in the range 140 to 150° C, partitions to the extent of 1 : 4 between 95 % methanol and hexane and is not precipitated by digitonin. These properties together with its infrared absorption spectrum suggest the compound may be a saturated xanthophyll.

Fractions IIa and IIb are not readily separated from one another on alumina. When either one was isolated and rechromatographed, small amounts of the other were obtained by rechromatography. This behavior suggested that the two may be isomers. Similar behavior was noted during the chromatographic purification on alumina of phytadiene as well as with farnesene.

Because of the apparently reversible conversion of IIa to IIb and because of the difficulty of obtaining large amounts of either material due to instability the two compounds were not in general separated from one another for the further studies.

The ultraviolet spectrum of fraction II, which shows a characteristic maximum at 231 mμ (specific abs. coefficient = 55.4), indicates the presence of a pair of conjugated double bonds. The theoretical absorption maximum for a compound of this type is, according to Woodward's rule (9), at 227 mμ, but shifts of several mμ have been found by Woodward and by O'Connor and Goldblatt (4) for various substituted dienes. Non-conjugated systems do not exhibit appreciable absorption above 210 mμ, and trienes may be distinguished by their three maxima in the 260 mμ to 280 mμ region.

The infrared spectrum of fraction II furnishes evidence that the compound is of an isoprenoid nature. In the fingerprint region of 4,000 cm^{-1} to 1,000 cm^{-1} the peaks characteristic of conjugated diene terpenes are matched very closely in position and intensity by the maxima of the fraction II spectrum. It may be particularly noted that the infrared spectrum of fraction II exhibits a wide band at 1,600 cm^{-1} corresponding to that expected of a compound containing both conjugated and non-conjugated double bonds. A prominent peak at 800 to 840 cm^{-1} is consistent with the presence in the compound of a propylidine group. A further peak at 890 cm^{-1} corresponds to that expected of a compound containing a methylene group.

Molecular weight determination by the Signer method (Truesdail Labs.), yielded duplicate values of 297 and 284. The average weight of 290.5 is in reasonable agreement with that expected for a C_{20} isoprenoid compound (expected—272).

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN CAROTENE AND RELATED FRACTIONS OF TOMATO FRUITS INJECTED WITH 2-C^{14}-MEVALONIC ACID WHILE IMMATURE AND INCUBATED FOR PERIODS INDICATED

FRACTION	FRUIT INCUBATED 1 DAY HARVESTED GREEN	FRUIT INCUBATED 6 DAYS HARVESTED GREEN	FRUIT INCUBATED 12 DAYS HARVESTED DARK PINK	FRUIT INCUBATED 17 DAYS HARVESTED DARK PINK
Crude phytoene	50.5	2.2	2.8	4.3
Phytofluene)	)	(	42.2	7.3
)	10.6	68.7 (		
Neo β-carotene)	)	(	4.6	3.7
β-carotene	1.5	9.5	25.6	38.8
ζ-carotene *	6.8	...	21.2	20.2
Minor pigments +	...	...	2.0	0.9
γ-carotene	...	...	0.2	0.6
Lycopene	...	...	0	0.4

Results expressed as activity in each fraction as a per cent of the total activity in the non-saponifiable fraction.
* Contains radioactive sterols.
\+ Probably a mixture of several pigments (8).
− No carotenes detected spectrophotometrically.

Samples of radioactive fraction II, containing principally fraction IIa but small amounts of fraction IIb, were degraded by ozonolysis. A radioactive volatile fragment was trapped in 2,4-dinitrophenylhydrazine reagent. The derivative was isolated and chromatographed on Whatman No. 3 paper, using the solvent systems: 5% ether: 95% hexane, and 80% ethanol: 20% hexane. The behavior of the compound in these solvents was found to be identical with that of authentic 2,4-dinitrophenylhydrazone of acetone. The recovery of the 2,4-dinitrophenylhydrazone of acetone was, however, low due no doubt to loss of the volatile degradation product during ozonolysis.

From the non-volatile ozonolysis residue relatively large amounts of labeled levulinic acid and labeled malonic acid were isolated by chromatographic procedures. These were identified by their chromatographic behavior on Whatman No. 1 paper in the five solvent systems: *n*-propanol 6-conc. ammonium hydroxide 4; ethanol 30-water 15-conc. ammonium hydroxide 5; ether 13-acetic acid 3-water 1; *n*-butanol 1-pyridine 1-water 1; and water saturated with *n*-butanol 95-formic acid 5. All attempts to determine the specific activities of the three labeled ozonolysis fragments of fraction II were unsuccessful due to the large inaccuracies involved in measurement of the small amounts of materials involved.

An isoprenoid structure which is derived from 2-C^{14}-mevalonic acid without randomization, should yield radioactive acetone, levulinic acid, and malonic acid upon ozonolysis and which would also be expected to possess other known properties of fraction II is proposed in figure 1. A comparison of the expected yields of radioactivity in the various ozonolysis fragments of the structure shown in figure 1 with those found in two separate degradation experiments are presented in table IV. These yields are low as yields from such degradation are often found to be.

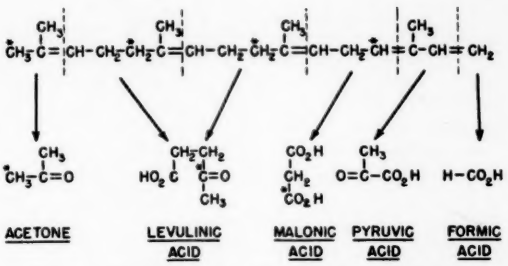

FIG. 1. Proposed structure of fraction II showing expected position of the label from 2-C^{14} mevalonate in fraction II and its ozonolysis products.

The structure of figure 1 should yield on degradation in addition to labeled acetone, levulinic acid and malonic acid, unlabeled pyruvic acid and an unlabeled oxidation product of the terminal methylene group. The terminal methylene group was determined by periodate-permanganate oxidation to formaldehyde (2) on a sample consisting entirely of fraction IIa. The yield of formaldehyde amounted to 79.2% of that expected on the basis of the structure of figure 1. The presence of unlabeled pyruvic acid among the ozonolysis products has however not been rigorously authenticated.

TABLE IV

EXPECTED (ON BASIS OF STRUCTURE OF FIG 1) AND ACTUAL YIELDS OF RADIOACTIVITY IN OZONOLYSIS FRAGMENTS OF FRACTION II FORMED FROM 2-C^{14}-MEVALONIC ACID IN TOMATO FRUITS

	EXPECTED		FOUND	
	CPM	% TOTAL	CPM	% TOTAL
(a) SAMPLE OF FRACTION II CONTAINING 342,000 CPM				
Acetone	86,000	25	9,100	2.7
Levulinic acid	172,000	50	63,800	22.4
Malonic acid plus unknown	86,000	25	30,100	8.8
(b) SAMPLE OF FRACTION II CONTAINING 239,000 CPM				
Acetone	60,000	25	4,074	1.7
Levulinic acid	120,000	50	51,516	21.6
Malonic acid plus unknown	60,000	25	46,000	19.2

DISCUSSION

Indirect evidence has been obtained for the participation of fraction II in carotene synthesis. The compound is formed rapidly during fruit development and appears to relinquish its radioactivity to carotenes during fruit ripening. Degradative studies as well as spectroscopic and chromatographic information have led to the formulation of a possible structure for fraction II. These studies have, however, been hampered by the difficulty of accumulating large amounts of fraction II, which is found in tomato fruit to the extent of less than one to several micrograms per gm of fruit.

Lynen et al (3) have reported the conversion of mevalonic acid to Δ^3-isopentenol pyrophosphate units, which condense to form farnesyl pyrophosphate and subsequently, squalene. An analogous series of reactions may be suggested for the biosynthesis of the carotenes. In this case, four isopentenyl residues may be visualized as condensing to yield the 20 carbon analog of farnesyl pyrophosphate. According to this suggested pathway, fraction II would be synthesized from mevalonate via Δ^3-isopentenol pyrophosphate. One molecule of fraction II would condense upon the pyrophosphate form of a similar C_{20} intermediate, forming a C_{40} skeleton which would at the time of fruit ripening be dehydrogenated to yield one or another of the carotenes proper. The primary C_{40} compound has, however, not yet been discovered. This may be because it is further transformed within the fruit as rapidly as it is formed.

Summary

A substance has been isolated from tomatoes which appears to be an intermediate in the pathway from mevalonic acid to the carotenes. The results of infrared spectroscopy and degradative studies indicate a probable structure of 3, 7, 11, 15 tetramethyl-hexadeca-1, 3, 6, 10, 14 pentaene.

Acknowledgments

The authors wish to express their appreciation to the Spectroscopy Unit of the Instrumentation and Analysis Group, Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture for certain of the infrared analyses.

Literature Cited

1. Braithwaite, G. D. and T. W. Goodwin. 1957. Biosynthesis of Beta-carotene from DL-beta-hydroxy-beta-methyl-delta-2-C^{14} valerolactone by *Phycomyces blakesleeanus* and carrot slices. Biochem. Jour. 67: 13P–14P.
2. Lemieux, R. U. and E. von Rudloff. 1955. Periodate-permanganate oxidations. II. Determination of terminal methylene groups. Can. Jour. Chem. 33: 1710–1713.
3. Lynen, F., J. Knappe, H. Eggerer, U. Henning, and B. W. Agranoff. 1959. Biosynthesis of terpenes. Fed. Proc. 18: 278.
4. O'Connor, R. T. and L. A. Goldblatt. 1954. Correlation of ultraviolet and infrared spectra of terpene hydrocarbons. Anal. Chem. 26: 1726–1737.
5. Park, R. B. and J. Bonner. 1958. Enzymatic synthesis of rubber from mevalonic acid. Jour. Biol. Chem. 233: 340–343.
6. Purcell, A. E., G. A. Thompson, Jr., and J. Bonner. 1959. The incorporation of mevalonic acid into tomato carotenoids. Jour. Biol. Chem. 234: 1081–1084.
7. Tavormina, P. A., M. H. Gibbs, and J. W. Huff. 1956. The utilization of beta-hydroxy-beta-methyl-delta-valerolactone in cholesterol biosynthesis. Jour. Amer. Chem. Soc. 78: 4498–4499.
8. Trombly, H. H. and J. W. Porter. 1953. Additional carotenes and a colorless polyene of Lycopersicon species and strains. Archiv. Biochem. Biophys. 43: 443–457.
9. Woodward, R. B. 1942. Structure and absorption spectra. III. Normal and conjugated dienes. Jour. Amer. Chem. Soc. 64: 72–75.

SOME QUANTITATIVE CONSIDERATIONS IN IRON NUTRITION OF HIGHER PLANTS [1]

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Among the essential elements taken up by the roots none offers as troublesome a problem as iron with respect to its solubility in aqueous solutions. From the equilibrium constants for the reactions of iron ions with hydroxyl ions (11) it is apparent that the ferric iron concentration drops below one part per billion between the pH values of 4 and 5, and that it decreases by a factor of 1,000 for each unit increase of pH value. Ferrous iron is not very susceptible to precipitation as the hydroxide. However, in aqueous solutions in the presence of oxygen ferrous iron is thermodynamically unstable. Under atmospheric oxygen pressure the ferric ion is more stable in acid solutions, while at a basic pH the equilibrium favors ferrous iron (13). In order that ionic iron supply sufficient iron to the plants it is necessary that the minimum concentration required for adequate growth be no higher than the saturation concentration given by the solubility product. At a pH of 9 the saturation concentration of both cationic forms of iron drops below 10^{-20} M/liter in a solution in equilibrium with atmospheric oxygen. Since on many occasions plants do not suffer from iron deficiencies when growing in soils having such high pH values, it may be possible that iron is taken up by plants through exchange phenomena or complex formation. Evidence for the importance of some contact effects between roots and iron compounds has been demonstrated by Eaton (3) and subsequently confirmed by others (2, 5, 17).

The present paper deals with a comparison between the thermodynamically possible saturation concentration and the minimum required concentration of iron ions in nutrient solutions. In this study inorganic compounds of iron and iron ethylenediaminetetraacetate (FeEDTA) have been used.

METHODS AND MATERIAL

I. GENERAL METHOD FOR GROWING PLANTS: The plants as indicated below were germinated in sand. After they reached a suitable size, usually after full development of the cotelydons, they were transplanted into the nutrient solution. Aerated Hoagland solution No. 1 was used, to which the usual amounts of micronutrients were added (7). Since recent investigations show that chlorine may be essential (1), 3 ppm of this element as KCl were added. The micronutrient stock solutions were especially purified by the method of Stout and Arnon (16). Iron was supplied as FeEDTA (10), ferric oxide, ferrous sulfate, or ferric chloride. Crocks or tanks, which were used as growing containers, and lids were painted with asphaltum varnish or with Amercoat paint (Amercoat Co.). Prior to each experiment lids and crocks were washed in dilute HCl and rinsed with distilled water. Samples of recently matured leaves were weighed separately after harvest, washed in approximately 2 % Dreft (Proctor and Gamble) solution and rinsed in distilled water. The samples were dried at 65° C with forced ventilation. The dried samples were ground in a glass mortar with glass pestle and stored in glass jars. Contact with iron containing substances was avoided.

II. DESCRIPTION OF EXPERIMENTS: *A. Minimum iron concentration*: Buckwheat, *Fagopyrum esculentum* (Moench); Corn, *Zea Mays* (L); Cotton, *Gossypium sp.* (L); Lupine, *Lupinus sp.* (L); Pea, *Pisum sativum* (L); Sunflower, *Helianthus annuus* (L); Tobacco, *Nicotiana tabacum* (L); and Tomato, *Lycopersicum esculentum* (Mill) were grown from July 1 until July 25 in 40 liter crocks, containing solutions at about pH 6. Iron was supplied as FeEDTA at 0.00, 0.01, 0.05, 0.25, and 1.00 ppm. Each crock contained three plants. The solutions were exchanged at intervals of 7 days initially and of 3 days near the end of the experiment.

Buckwheat, corn, cotton, sunflower, and tobacco were grown at pH 4.5 for five weeks in 100 liter tanks. At intervals of 1 or 2 days the solutions were analyzed and the iron concentrations maintained at 10. 60, and 160 part per billion (ppb) through addition of proper amounts of ferrous sulfate.

B. Manganese-iron ratios: Sunflower and corn plants were grown in nutrient solutions corresponding to half strength Hoagland solution from July 30 until August 22. The sunflower plants were grown singly at 1.25, 0.25, 0.02, and 0.005 ppm Fe as FeEDTA in 40 liter crocks. At each iron level five manganese levels were chosen in such a way that the following Mn-Fe ratios could be investigated: 0.003, 0.03, 0.3, 3, and 30. Corn plants were grown singly in 40 liter crocks at 5, 1, and 0.2 ppm Fe as FeEDTA. At each iron level manganese was added to produce the following Mn-Fe ratios in the nutrient solutions: 0.001, 0.01, 0.1, 1, and 10.

C. Effects of pH: Sunflower plants were grown in 15 liter containers at solution pH values from 3.5 to 8.5 between June 3 and June 26. The pH was adjusted once or twice per day with 1 *N* H_2SO_4 or 1 *N*

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KOH. Three plants were used per container. Ten ppm of iron were supplied as Fe_2O_3, $FeCl_3$, FeEDTA. A series without iron served as control.

In an additional experiment the pH effect on iron supplied as the ferrous sulfate was investigated. Three corn plants were grown per 100 liter tank at pH 4.5, 5, 5.5, and 6.0. The pH was adjusted daily and the fluctuations stayed mostly within 0.2 pH unit. At the beginning of a 3 week growing period 5 ppm iron were added to the solutions which, in this case, were made with technical grade salts. Similar experiments were conducted with cotton, tobacco, buckwheat, and sunflower.

III. Analytical Methods: The iron was determined colorimetrically by the ortho-phenanthroline method, after the samples had been digested with redistilled concentrated sulfuric acid and iron free hydrogen peroxide (9). For determining ionic iron in nutrient culture solutions use was made of 4:7-diphenyl-1, 10-phenanthroline (14).

For chlorophyll determinations, discs were punched from the leaves with a brass cork borer prior to washing. The discs, generally 1 to 2 gm were ground in washed sand, a few milliliters of acetone and a pinch of calcium carbonate. Chlorophyll was then extracted with 80 % (v/v) acetone. The absorption of the extract was determined with a Beckman Model B spectrophotometer at 6450 A and 6630 A. The chlorophyll concentrations were calculated with help of the specific absorption coefficients published by Mackinney (12).

The total nitrogen was determined by digesting 25 to 50 mg of plant material in a micro-Kjeldahl flask with 0.5 g of digestion mixture (made by grinding 20 g HgO with 250 g K_2SO_4), a few grains of emery (60 mesh), and 1.5 ml concentrated sulfuric acid.

The soluble nitrogen was determined by heating about 50 mg of plant material in 10 ml of 0.05 M acetic acid at 80° C for 10 minutes. After cooling and centrifuging the samples were filtered into a micro-Kjeldahl flask containing about 100 mg of hydrogen-reduced iron powder and a few grains of emery. The extraction procedure was repeated two more times by shaking the sample for 5 minutes with 5 ml aliquots of 0.05 M acetic acid and then centrifuging them for another 5 minutes. 2.5 ml of 3.6 *N* sulfuric acid were added to the extract and the flasks were shaken occasionally for 15 minutes. This procedure insured reduction of nitrates. One drop of antifoaming agent (Foamex) was added and the extracts were boiled gently for 5 minutes. 1.5 ml concentrated H_2SO_4 and 0.5 g of the digestion mixture were added and the samples digested. At the end of the digestion period the samples were boiled for a few minutes with 3 ml of 6 to 7 *N* HCl.

The samples were transferred to the micro-Kjeldahl distillation apparatus, 10 ml of 10 *N* NaOH containing 75 g $Na_2S_2O_3 \cdot 5H_2O$ per liter were added and the samples steam distilled into 2 % boric acid. The boric acid was then titrated with 0.010 *N* HCl.

All data are expressed on an air-dry basis.

Results

I. Minimum Iron Concentration: *a. Fe supplied as FeEDTA.* The yields, iron and chlorophyll analyses of corn, pea, sunflower, and tobacco are presented in table I. Apparently the plants respond differently to the iron treatment, the requirement being 10 to 100 times higher for corn than for tobacco and pea while sunflower plants lie between these extremes. Other data not presented here indicate that the iron requirement for lupine was similar to that of corn; that of buckwheat, cotton, and tomato were more comparable with sunflower. In spite of these different iron concentration requirements the correlations between amount of iron and chlorophyll in plants are similar for most species studied (fig 1). From the appearance of iron deficiency symptoms it could be observed that the iron requirement was higher immediately following the transfer into the nutrient solutions than during later stages of development.

A. *Iron supplied as an inorganic ion.* Due to the precipitation of ferric hydroxide the iron concentrations were subject to considerable fluctuations. In the case of corn, for example, the iron concentrations varied from 5 to 16, 32 to 45, and 59 to 161 ppb, respectively, for the desired concentrations of 10, 40, and 160 ppb. 10 ppb Fe was definitely not sufficient for corn. After some initial chlorosis corn produced vigorous plants at 40 ppb. At 40 and 160 ppb the final iron and chlorophyll contents of the leaves were 90 to 100 ppm and 1.1 to 1.2 %, respectively. For

Table I

Effect of Iron Concentration on Corn, Pea, Sunflower, and Tobacco*

	Fe added ppm	Dry wt g	Fe in leaves ppm	Chlorophyll %
Corn	0.00	1.0	20	0.02
	0.01	1.0	19	0.07
	0.05	0.8	24	0.09
	0.25	0.9	21	0.22
	1.00	1.8	26	0.31
Pea	0.00	1.3	14	0.03
	0.01	2.3	16	0.07
	0.05	4.5	51	0.65
	0.25	5.0	76	0.99
	1.00	5.6	101	0.99
Sunflower	0.00	1.9	15	...
	0.01	2.9	15	0.01
	0.05	16.7	35	0.47
	0.25	60.5	61	0.75
	1.00	80.1	67	0.79
Tobacco	0.00	1.6	20	0.001
	0.01	4.0	20	0.17
	0.05	38.0	64	0.83
	0.25	64.7	86	1.07
	1.00	38.2	117	1.06

* Iron supplied as FeEDTA.

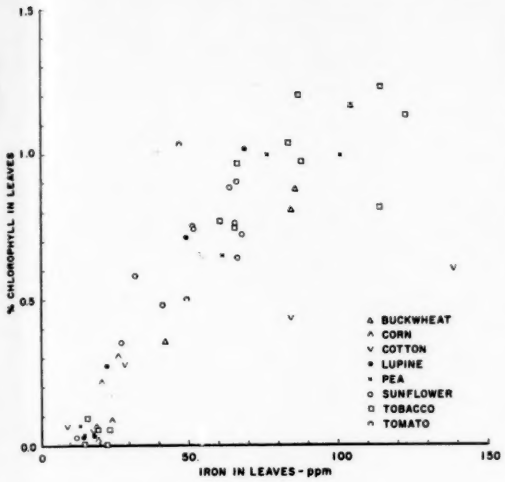

FIG. 1: Iron-chlorophyll relations in leaves of various plants.

sunflower, tobacco, buckwheat, and cotton, a concentration of 10 to 40 ppb was sufficient throughout the experiment.

II. MANGANESE-IRON RATIOS: A change in the iron concentration brings about a change in the ratio of iron to other nutrients. It has been suggested that the ratio of manganese to iron may be important in causing chlorosis (15). As determined by dry matter accumulation, optimum growth with sunflower plants was obtained at a manganese iron ratio of 0.03 (table II). In general, these plants also showed the highest chlorophyll content. The iron relations in the leaves were less clear. At high levels of iron most iron was found at a Mn-Fe ratio of three; at lower levels of iron the relatively highest concentration of iron in plants was at a lower ratio than three.

Table II indicates that for corn no optimum manganese-iron ratio was found within the range investigated; the highest yields were obtained with the lowest manganese concentration.

III. EFFECTS OF PH: The hydrogen ion concentration has an important effect on the solubility of iron. It may thus be possible to investigate a range of iron concentrations by varying the pH value and adding a sufficient amount of iron to the solution to permit a saturated solution during the experiment. Within the tested pH range from 3.5 to 8.5, 10 ppm iron as ferric oxide was ineffective and essentially no growth was observed above the control without iron (table III). With ferric chloride as iron supply the yields decreased from pH 4.5 to 8.5. A corresponding decrease in iron and chlorophyll content was found. With FeEDTA no clear effect of the pH was observed over the pH range 4.5 to 8.5. At pH 3.5 the yields were much reduced in the FeEDTA and the ferric

TABLE II

EFFECT OF MANGANESE-IRON RATIO ON SUNFLOWER AND CORN*

SUNFLOWER						CORN					
Fe IN SOLUTION PPM	Mn/Fe					Fe IN SOLUTION PPM	Mn/Fe				
	3×10^{-3}	3×10^{-2}	3×10^{-1}	3×10^{0}	3×10		10^{-3}	10^{-2}	10^{-1}	10^{0}	10
	Dry wt g						*Dry wt g*				
1.25	17.3	22.2	15.5	13.1	8.4	5.0	4.8	3.1	2.5	0.03	2.4
0.25	14.3	12.2	13.9	11.5	9.1	1.0	2.8	3.0	0.3	0.54	0.5
0.05	12.3	7.3	11.9	4.3	1.6	0.2	1.6	0.4	1.6	0.4	0.3
0.02	3.4	4.9	1.2	0.9	1.1						
0.005	0.7	1.1	1.3	0.6	0.8						
	Iron content in leaves ppm						*Iron content in leaves ppm*				
1.25	67	71	81	82	41	5.0	37	30	33	dead	41
0.25	56	56	58	78	53	1.0	(21)	26	(42)	41	21
0.05	34	33	33	29	24	0.2	23	29	(24)	dead	(29)
0.02	20	29	27	20	25						
0.005	17	(17)**	14	17	(12)						
	Chlorophyll content %						*Chlorophyll content %*				
1.25	0.91	1.10	0.98	0.98	0.51	5.0	0.62	0.50	0.46	dead	0.28
0.25	0.91	0.78	0.75	0.80	0.50	1.0	0.25	0.23	0.19	0.26	0.19
0.05	0.29	0.41	0.37	0.37	0.09	0.2	0.22	0.36	0.12	dead	0.11
0.02	0.14	0.36	0.06	0.03							
0.005	*Necrotic, not analyzed*										

* Iron supplied as FeEDTA.
** ( ) analyses uncertain because of insufficient amounts of plant material.

TABLE III

EFFECT OF pH AND IRON SOURCE ON SUNFLOWER

TREATMENT		DRY WT G	Fe IN LEAVES PPM	CHLOROPHYLL %	TOTAL N %	SOLUBLE N % OF TOTAL
Fe	pH					
Fe_2O_3						
	3.5	1.0	16		Not analyzed	
	4.5	1.6	15		4.3	38
	5.5	2.3	19		5.3	25
	6.5	1.9	19	***	4.9	27
	7.5	2.4	11		5.7	31
	8.5	1.8	12		4.5	29
$Fe\ Cl_3$						
	3.5	6.6	201	0.71	6.6	14
	4.5	45.0	200	0.97	6.4	17
	5.5	41.7	129	0.84	6.1	17
	6.5	40.5	138	0.83	6.4	16
	7.5	35.9	50	0.63	4.8	23
	8.5	16.0	32	0.44	4.3	18
FeEDTA						
	3.5	4.6	149	0.89	6.4	18
	4.5	44.1	95	0.93	6.6	10
	5.5	28.4	102	0.93	7.1	19
	6.5	34.2	93	0.91	6.2	16
	7.5	49.9	109	1.00	6.6	11
	8.5	34.8	84	0.87	5.9	7
No iron						
	3.5	1.1	10		6.9	49
	4.5	1.6	10		6.3	26
	5.5	1.8	9		5.5	34
	6.5	1.7	7	***	6.2	29
	7.5	2.4	5		5.3	29
	8.5	2.3	9		4.7	31

*** Extreme chlorosis, not analyzed for chlorophyll.

chloride series. The iron contents in the leaves of these plants were high. It is probable that the reduced yield at this pH is a direct effect of the hydrogen ion concentration.

In an additional experiment iron was supplied as ferrous sulfate to corn plants. At pH 5 and higher, corn plants were chlorotic. Dry weights, chlorophyll and iron contents decreased with increasing pH (table IV). Other experiments not presented here gave similar results for cotton, tobacco, buckwheat, and sunflower, however, corn seemed to show the deficiency symptoms earlier than the other plants. This again indicates that corn requires a higher iron concentration.

There appeared to be some relation between the amounts of acid or base required to maintain a constant pH and the iron nutrition of the plant. In a typical case, three sunflower plants, approximately one month old, growing in a 40 liter crock required the addition of 22 meq H_2SO_4 to maintain the pH at six over a period of 12 days when the solution contained 1 ppm Fe as FeEDTA. When the solutions contained no added iron 28.5 meq KOH were required to maintain the same pH over the same period of time. It therefore appears that plants in Hoagland nutrient solutions tend to make the solution more basic if iron is present and more acid if iron is absent. The disturbances caused within the plant through the absence of iron apparently affect the uptake of nutrients

TABLE IV

EFFECT OF pH ON CORN*

pH	FRESH WT G	CHLOROPHYLL %	IRON PPM
4.5	20.3	0.96	102.9
5.0	21.9	0.65	55.2
5.5	9.7	0.39	36.3
6.0	8.9	0.38	21.6

* Initial iron concentration 5 ppm as ferrous sulfate.

from solutions. One possible disturbance is reflected in table III. The total nitrogen content in iron deficient plants is slightly smaller while the fraction of soluble nitrogen is considerably higher than in healthy green plants.

Discussion

I. Minimum Iron Concentration: Our data indicate that the minimum concentration of ferrous or ferric iron required for normal plant growth is many times higher than the thermodynamically possible saturation concentration. At neutral pH, for example, the equilibrium iron concentration in a culture solution is about a billion times lower than the iron requirement for plants. A theory explaining iron uptake from sparingly soluble iron compounds releasing ferric ions is therefore inadequate. Ferrous iron is highly soluble in water but is unstable with respect to oxidation to the ferric state. Oxidation should occur more rapidly under acid conditions, but, due to the special path of reaction, the rate of reaction is very slow under acid conditions (11). It is unlikely that soils are sufficiently acid for this to occur. In a Hoagland solution the rate of iron precipitation from ferrous sulfate is appreciable at pH 4 and increases rapidly as the pH is increased to 4.5 or 5. (13). The hydrogen ion concentration of almost all soils is therefore favorable for rapid oxidation of ferrous iron. At equilibrium the iron in soils is largely present in the oxidized state. It is impossible to reduce more iron than determined by the equilibrium conditions. A lower oxygen tension, however, would shift the equilibrium position in favor of ferrous iron. To obtain an appreciable shift the oxygen tension would have to be reduced to such an extent that oxygen deficiency might be a serious problem for many plants.

The magnitude of the iron concentration required for good plant growth was found to depend on various factors such as plant species, form of iron, and effect of other nutrients.

Similar correlations between chlorophyll and iron in leaves were obtained (fig 1). Apparently, the internal requirements for the various species are similar. Differences in requirement for iron in the nutrient solution may thus be connected with the roots, probably with the ability to take up iron. Figure 1, further indicates that plants have a basic iron requirement of around 20 ppm before any chlorophyll is formed.

Provided the concentration is maintained, iron as ferrous sulfate or ferric chloride satisfies the nutrient requirement of plants at concentrations 1/10th or less than that of iron supplied as FeEDTA. Although opinions on the uptake of chelated iron differ (18, 19). a less rapid uptake of the chelated iron would be expected if iron is taken up as a chelate or by an exchange reaction on the root surfaces. If taken up in the chelated form, the larger molecule size would probably result in a slower rate of uptake or at least a slower rate of diffusion to the root. If the chelate releases the iron before the uptake reaction, uptake occurs from the ionic form with a concentration at best equal to the initial ionic supply.

Ferric oxide and ferric chloride should both have produced solutions saturated with iron. The pronounced differences in yield (table III) indicate that equilibrium was not established rapidly, if at all. Probably not enough iron dissolved from the ferric oxide to bring about saturated solutions, since at pH 3.5 sufficient iron could have dissolved to produce growth. Ferric hydroxide did not precipitate fast enough from ferric chloride and plant growth was observed over the entire pH range under investigation. The iron chlorophyll relations in sunflower leaves depend but little on the source of iron.

Chlorosis occurred readily when the ratio of manganese to iron was kept constant but the absolute concentrations were lowered; therefore, the Mn-Fe ratio (15) alone does not govern iron chlorosis. The iron-chlorophyll relations in the plants depend little, if at all, on the iron manganese ratio of the nutrient medium. Therefore, theories which involve the precipitation of iron in the leaves through manganese can hardly explain the manganese induced chlorosis. An increased iron content of the roots, caused by a high manganese concentration on the clay exchange complex was found by Epstein (4). Since the iron content in tops was lower, he concluded that the precipitation must have occurred within the roots. Analysis of roots is difficult to interpret since iron may have precipitated on the root surface. Hewitt (6) has pointed out that chemical potentials should not be applied to plant cells as if one were dealing with aqueous solutions to explain the effect of manganese on iron chlorosis.

II. Some Considerations on Stability of FeEDTA: The addition of FeEDTA to a nutrient culture solution leads to numerous possible chemical reactions (13). The equilibrium conditions for the most important chelation reaction of iron with EDTA can be formulated as follows:

$$(FeEDTA^{-}) = K\ (EDTA^{-4})\ (Fe^{+3})$$

K represents the chelating constant, and the expressions in parenthesis the activities or an approximation of the concentrations of the respective compounds.

EDTA dissociates into various anions and the relative amount of the four-valent anion depends on the pH. Also, the maximum concentration of ferric iron depends on the pH. Should the term (Fe^{+3}) exceed the saturation concentration of iron, ferric hydroxide will precipitate and more $FeEDTA^{-}$ will dissociate. Calculations (13) lead to the conclusion that with 5 ppm Fe as FeEDTA in an aqueous solution the precipitation of iron will start between pH 5 and 6, but will be negligible with rising pH until a pH between 9 and 10 is reached. Different initial concentrations of FeEDTA as used in our experiments cause only negligible changes from these values.

EDTA chelates many of the cations present in a nutrient culture solution and the equilibrium conditions can be formulated in the same way as for iron. The chelation constant is highest for iron. Since

the value of $EDTA^{-4}$ is identical for all chelation reactions in a culture solution, the product of the chelating constant with the concentration of the respective cation determines whether a cation is chelated preferentially. Because of the extremely low concentrations of ferric iron at high pH values most of the copper and zinc in a Hoagland solution containing 5 ppm Fe as FeEDTA should be chelated between pH 6 and 7. Between pH 7 and 8 manganese, calcium, and magnesium can cause serious interferences. An increase in the concentration of any of the above ions will lower the pH value at which this nutrient should become chelated preferentially to iron.

The above considerations are based on equilibrium conditions and for this reason must be applied with considerable caution. For example, even at a pH of 8.5 healthy plants could be obtained using FeEDTA as a source of iron in a Hoagland solution indicating that some of the reaction rates might have been slow.

III. Effect of Iron Deficiency on pH of Nutrient Solutions: Changes of the pH value in a nutrient culture solution are primarily caused by differential uptake of anions and cations. Nitrate was the most abundant ion in our culture solution. Since it is absorbed rapidly the pH rises under normal conditions. With iron starvation the pH drops, indicating an excess of cations absorbed. Table III shows high soluble and low total nitrogen contents in chlorotic leaves. Similar results were found by Iljin (8). This suggests that iron is directly or indirectly involved in the nitrogen metabolism of plants. As a consequence soluble nitrogen may accumulate and hinder the uptake of more nitrate in iron deficient plants.

Summary and Conclusions

Buckwheat, corn, cotton, lupine, pea, sunflower, tobacco, and tomato were grown in nutrient culture solutions at different levels of iron. Although the iron was maintained in sufficient absolute quantities at low concentrations, the plants showed symptoms of iron deficiencies, thus indicating an existence of a minimum required concentration for adequate plant growth. This minimum iron concentration is far higher than the thermodynamically possible saturation concentration over practically all of the pH range, hence, the uptake of iron in soils must occur other than from sparingly soluble ionic iron. It was shown that the minimum iron concentration depended on the plant species, the form of iron supplied, and the ratio to other nutrients. It appears, however, that the metabolic or internal requirement for iron in plants, as judged by the iron chlorophyll relationships, is mostly independent of the species, form of iron, and ratio to other nutrients.

Iron deficient plants take up a relatively smaller amount of anions (nitrate) than cations. This may be interpreted as evidence for a function of iron in nitrogen metabolism.

Literature Cited

1. Broyer, T. C., A. B. Carlton, C. M. Johnson, and P. R. Stout. 1954. Chlorine—A micronutrient element for higher plants. Plant Physiol. 29: 526–532.
2. Chapman, H. C. 1939. Absorption of iron from finely ground magnetite by citrus seedlings. Soil Sci. 48: 309–315.
3. Eaton, F. M. 1936. Automatically operated sand-culture equipment. Jour. Agr. Research 53: 433–444.
4. Epstein, E. 1950. The micronutrient cations iron, manganese, zinc, and copper: their uptake by plants from the adsorbed state. Ph.D. Thesis, University of California, Berkeley.
5. Guest, P. L. 1944. Root-contact phenomena in relation to iron nutrition and growth of citrus. Proc. Amer. Soc. Hort. Sci. 44: 43–48.
6. Hewitt, E. J. 1948. Relation of manganese and some other metals on the iron status of plants. Nature 161: 489–490.
7. Hoagland, D. R. and D. I. Arnon. 1950. The water-culture method for growing plants without soil. Cal. Agric. Exp. Station, Circular 347.
8. W. S. Iljin. 1951. Metabolism of plants affected with lime-induced chlorosis (calciose) I. Nitrogen metabolism. Plant and Soil III: 239–256.
9. Jacobson, L. 1945. Iron in the leaves and chloroplasts of some plants in relation to their chlorophyll content. Plant Physiol. 20: 233–245.
10. Jacobson, L. 1951. Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. Plant Physiol. 26: 411–413.
11. Latimer, W. M. 1952. Oxidation potentials. 2nd ed. Prentice Hall, New York.
12. Mackinney, G. 1941. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. 140: 315–322.
13. Oertli, J. J. 1956. Studies in the uptake of iron by higher plants. Ph.D. Thesis, University of California.
14. Smith, G. F., H. W. McCurdy, Jr., and H. Diehl. 1952. The colorimetric determination of iron in raw and treated municipal water supplies by use of 4:7 diphynyl-1: 10-phenanthroline. Analyst 77: 418–422.
15. Somers, I. I. and J. W. Shive. 1942. The iron-manganese relation in plant metabolism. Plant Physiol. 17: 582–602.
16. Stout, P. R. and D. I. Arnon. 1939. Experimental methods for the study of the role of copper, manganese, and zinc in the nutrition of higher plants. Amer. Jour. Bot. 26: 144–149.
17. Stromme, E. R. 1951. Availability to plants of iron and manganese in glassy fruits. Ph.D. Thesis, Michigan State College.
18. Tiffin, L. O. and J. C. Brown. 1959. Absorption of iron from chelate by sunflower roots. Science 130: 274–275.
19. Wallace, A. and C. P. North. 1953. Lime induced chlorosis. California Agriculture 7: (8) 10.

DEMONSTRATION OF TWO TRANSLOCATION MECHANISMS IN STUDIES OF BIDIRECTIONAL MOVEMENT [1, 2, 3]

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INTRODUCTION

Bidirectional movement in phloem infers a passage upward and a simultaneous passage downward of one or more substances within the same section of stem. That this does occur was established with the use of radioactive tracers by Biddulph and Markle (3), Rabideau and Burr (6), Chen (4), and others. These researches, however, did not relate the movement to specific phloem bundles and therefore they were not useful in interpreting mechanisms of movement.

The ultimate objective of this kind of experimentation is, of course, to illustrate the mechanism by which minerals and metabolites are distributed through the circulatory system of the plant. A mechanism of movement based on the participation of the protoplast, as, for example, protoplasmic streaming, should be capable of sustaining a simultaneous bidirectional movement of two or more substances through the same phloem bundle or even through the same sieve tubes, whereas a pressure flow mechanism, based on turgor differences and resulting in a mass movement of substances, should preclude simultaneous bidirectional movement within the particular channel which conducts the flow. Therefore it should be possible to discriminate between the two kinds of mechanisms on the basis of reliable evidence for or against simultaneous bidirectional movement in individual phloem bundles. As will be seen later, evidence for two such mechanisms of translocation was obtained, and each mechanism appeared capable of supporting bidirectional movement. A number of people, in various states of seriousness, have suggested that perhaps a plant is capable of translocating materials in more than one way. To our knowledge the present study is the first concrete demonstration that two mechanisms may be operative side by side.

METHODS

Red Kidney bean plants, grown to an age of 12 days from the time the hypocotyl straightened in an aerated half strength Hoagland solution (with micronutrients), served as experimental material. The environmental conditions during growth and treatment were: temperature 23 ± 1° C; light, artificial fluorescent (two daylight + ten soft white tubes) on a 12-hour photoperiod, 1,000 to 1,200 ft-c; relative humidity, 60 ± 5 %. The growth room was protected from fallout radioactivity with high efficiency filters.

The translocation characteristics of ten individual plants were determined. In each experiment one of the plants was placed in a compartmentalized treatment chamber especially designed to effectively confine CO_2 tracer to a designated place over a single leaflet. The equipment has been described elsewhere (1). Two-hundred and fifty μc of C^{14} as $C^{14}O_2$ were then confined in a 1 inch diameter container over the upper surface of the first or lowermost trifoliate leaf. The CO_2 concentration in the container, of 5 ml volume, was approximately 1 % by volume. Five minutes later 50 μl of solution containing 100 μc of P^{32} as $NaH_2P^{32}O_4$ (pH 4) was sprayed onto a 1 inch diameter (approx.) area on the lower surface of the terminal leaflet of the second trifoliate leaf. The phosphorus concentration of the applied solution was 10 mM. A migration period of 30 minutes for the C^{14} tracer (25 min for the P^{32} tracer) was allowed. Previous experimentation had shown that within this time interval a portion of the C^{14} tracer would ascend, and a portion of the P^{32} tracer would descend, through the intervening internode. All experiments were performed between the 4th and 5th hours after the beginning of the daily photoperiod.

Immediately after the close of the migration period a 5 inch section of the stem was removed from the plant, the bark slit longitudinally, and stripped from the wood (2). Both wood and bark were immediately frozen in ether at −80° C and then dehydrated in vacuo at −5° C. After this they were partially rehydrated by exposure for 1 hour to air saturated with water vapor, then carefully flattened between smooth papers, weighted lightly and quickly dried in an oven at 85° C.

Three radioautograms were prepared from the tissues of each plant, one (B) showing the combined distribution of the C^{14} and the P^{32}, another (C) showing the distribution of only the P^{32}, and a third (A) showing the distribution of only the C^{14}. This was done as follows: the flattened tissues, cambial side out, were taped by their ends to an aluminum filter of sufficient thickness (27.6 mg/cm²) to absorb all (more than 99 %) of the C^{14} radiation, and the mounted tissue placed between two sheets of X-ray film. Panatomic film (Eastman Kodak Co.) has been used more recently and it has given better resolution. The unfiltered film (B), which received both the P^{32} and C^{14} radiation, was removed after an ex-

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[2] This investigation was made possible by a fellowship grant (to O. B.) from the Guggenheim Memorial Foundation, and supported in part by funds provided by the State of Washington, Initiative Measure No. 171.

[3] The radioisotopes were acquired from the U.S. A.E.C., Oak Ridge, Tenn.

posure period calculated from the radioactivity in the tissue to give satisfactory density. The film behind the filter (C), which received only that fraction of the P^{32} radiation energetic enough to penetrate the filter, was given an additional exposure of sufficient duration to compensate for the absorption (absorption factor = 1.48) and for the decay of the P^{32} during the extended period. The films were so arranged that B could be removed without disturbing the orientation of C with the tissue. After a decay period of at least ten P^{32} half-lives a third exposure (A) was made from the same position as film B to record only the C^{14} radiation.

The resolution of the images of the phloem bundles on films A and B was sufficiently good that direct visual comparisons could be made between the distribution of the combined tracers, C^{14} + P^{32}, and the C^{14} tracer only. A more satisfactory comparison, and therefore a better interpretation, was obtained by scanning radioautograms A and B across identical transects with a recording microdensitometer. The transects are indicated by the position of the dots on the margins of the radioautograms of figures 1 and 2. The resultant tracings, which were logarithmic on the ordinate, were superimposed so that by the difference in height of the tracings an indication of the P^{32} content of each phloem bundle was obtained (no quantitative interpretations were attempted). This procedure was necessary as the presence of the filter reduced the resolution of the P^{32} film to an unsatisfactory level for the localization of this tracer in the bundles.

The radioautograms were observed under 10 × magnification, so that the edges of each discernible strand could be located with a filar micrometer. Vertical lines were then erected on the microdensitometer tracings to test correspondence with the recorder's record. The lines were retained for those P^{32} laden bundles which were easily discernible (figs 3, 4, and 5).

The distribution of the tracers in the xylem was similarly studied from slit and flattened sections of the woody cylinder. However, such a small amount of tracer was found in the xylem that nothing of interest was gained by a study of this tissue except that the principal movement of the tracers was confined to the phloem during the migration periods employed.

Because of the significance of the results confirmation was sought in another manner. Consequently a different method of introducing another tracer, i.e., S^{35}, into the phloem was found. The simplest method of introduction, and one which rather surprisingly proved entirely satisfactory, consisted merely of placing S^{35}, as sulfate, into the nutrient medium of plants grown similarly to those above. Then after migration periods of 15 to 30 minutes, during which time the tracer ascended the xylem, the bark was stripped, frozen and dehydrated as above and radioautograms were prepared. The radioautograms showed that the S^{35} entered the bark predominantly at the nodes and moved within the phloem according to the pattern of movement which was in progress at the moment. The patterns displayed by this radioisotope corresponded in all essential details to those obtained in the former study. P^{32}, when used in the same manner as the S^{35}, gave similar results. In seeking confirmation in this manner we were dealing with but a single radioisotope and detecting the pattern of its occurrence in the phloem after it had entered this tissue predominately at the nodes. The essence of the confirmation is in the similarity of the patterns of distribution of the S^{35} and C^{14} tracers.

Results and Discussion

Information on several important aspects of the translocation process was obtained from the data: first, bidirectional movement occurred in all ten trials; second, there were two distinctly different patterns by which the bidirectional movement was accomplished; and third, the two patterns were sufficiently distinctive to require separate mechanisms for their production.

Within the ten trials there occurred an example, in relatively pure form, of each of the two translocation patterns. The majority of cases, however, showed combinations or mixtures of the two patterns indicating that the two mechanisms producing these patterns were frequently operative simultaneously, or during the same short period. Also there were variations in the amount of each tracer translocated through the internode in the ten trials. Variations occurred from predominantly C^{14} translocation from the first trifoliate leaf, with little export of P^{32} from the second, to predominantly P^{32} translocation from the second trifoliate leaf, with little export of C^{14} from the first. Again most of the trials were intermediate in the amount of each tracer translocated; it was from among these that the most usable examples were found for showing bidirectional movement. Such variability was also good evidence that export from a given leaf was sporadic rather than uniform.

The first pattern of translocation to be described involved the downward movement of P^{32} tracer in the phloem portion of the five (usually) leaf traces from the second trifoliate leaf which, in the bean, descend to the next node below before anastomosing with other bundles of the stem. The phloem intervening between these five bundles conducted C^{14} tracer upward from the node of the first trifoliate leaf. In this case, which is quite distinct from the next to be described, the bidirectional movement involved separate phloem bundles, some of which carried P^{32} tracer downward while others carried C^{14} tracer upward (figs 1 and 3).

The resolution of the phloem bundles in figure 1 should be carefully noted, for the diffuseness of the image of these bundles, in contrast to those in figure 2, indicates that the functional area of the phloem was relatively deeply embedded (from the cambium) in the bark tissue. This functional area would presumably correspond to the older, more mature region of the phloem bundle. The proximity of the tracer-

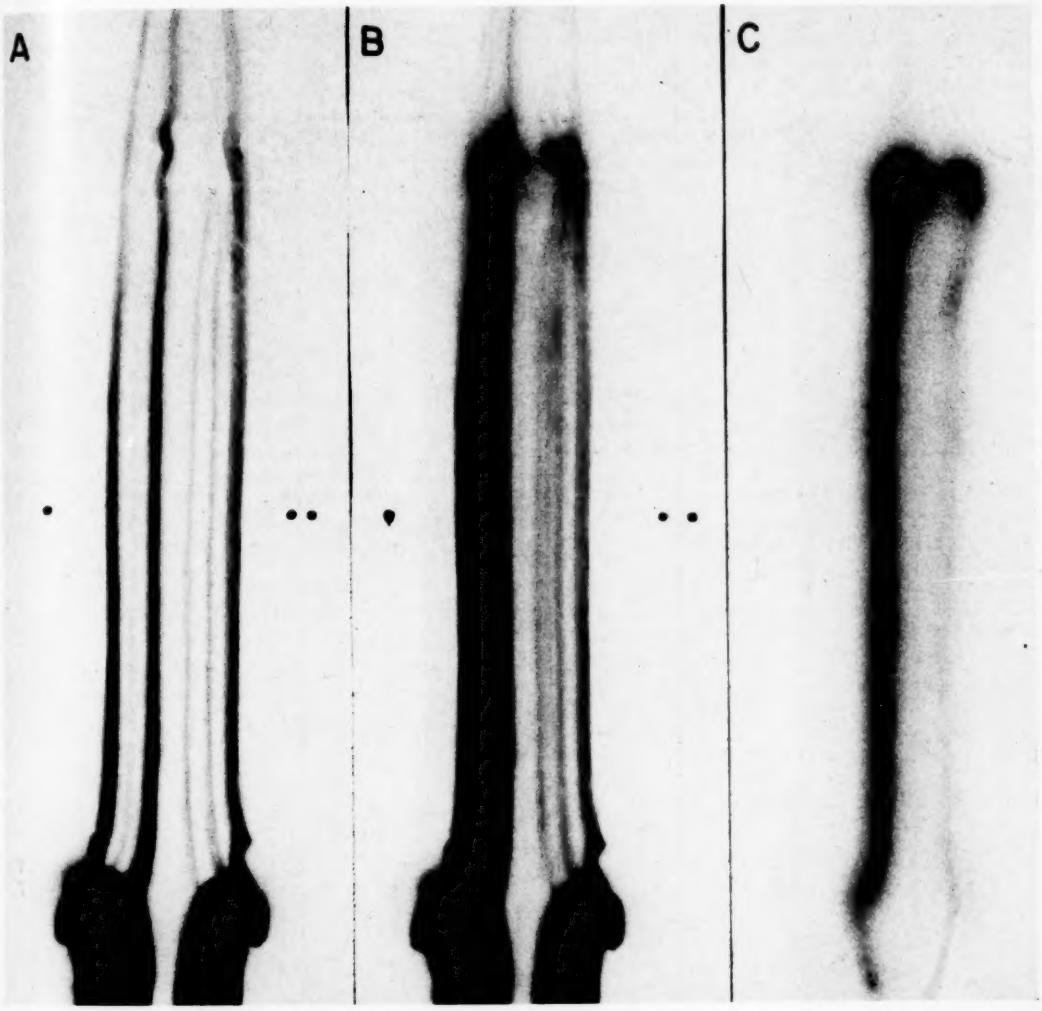

FIG. 1. Radioautograms of the same section of bark from a bean stem showing the location of the C^{14} (A), the C^{14} + P^{32} (B), and the P^{32} (C) tracers separately. The C^{14} tracer entered the stem from a leaf diverging at the lower node; the P^{32} from a leaf at the upper node. In A is shown the paths of the upward moving component of the C^{14} tracer. In C is shown (through a filter) the downward moving component of the P^{32} tracer. In B is shown one of the two ways in which the two tracers pass each other in the internode: they utilize separate phloem bundles. For details see text.

Exposure: A and B, 6 days 3½ hours; C, 12 days 3¼ hours. Magnification 2.8 ×.

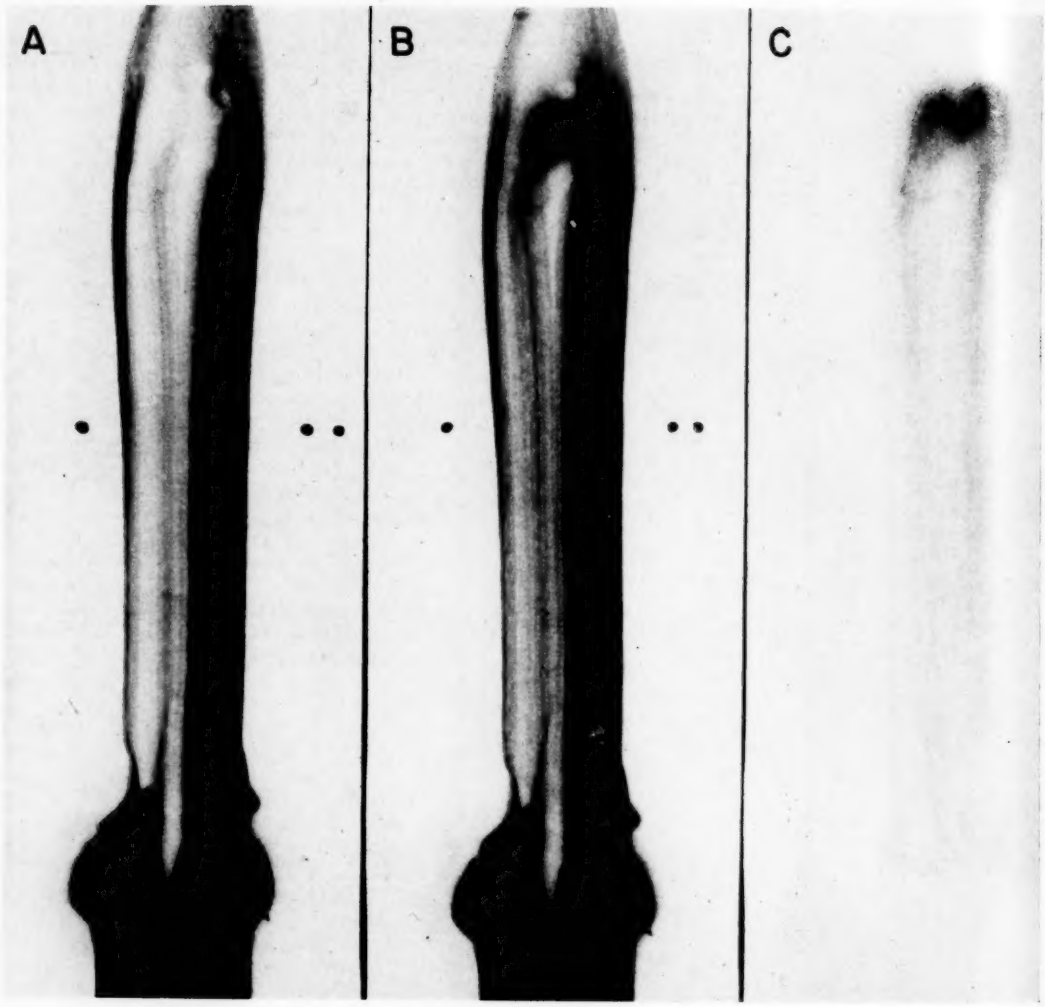

FIG. 2. Radioautograms of the same section of bark from a bean stem showing the location of the C^{14} (A), the C^{14} + P^{32} (B), and the P^{32} (C) tracers separately. The C^{14} tracer entered the stem from a leaf diverging at the lower node: the P^{32} from a leaf at the upper node. In A is shown the paths of the upward moving component of the C^{14} tracer. In C is shown (through a filter) the downward moving component of the P^{32} tracer. In B is shown a second way in which the two tracers pass each other in the internode; they utilize many of the same phloem bundles. For details see text.

Exposure: A and B, 14 days 3¾ hours; C, 39 days 2¼ hours.

Magnification 2.8 ×.

laden tissues to the film can be determined by the resolution of the radioautograms as all other factors influencing resolution have been held constant. However, detailed microradioautograms will be required to establish with certainty the position of the sieve tubes which participate in the conduction.

This pattern of movement corresponds to that which would be expected if a pressure flow mechanism of the Münch type were responsible for its production. The conduction, in this instance, was performed by older, more mature phloem cells located farthest from the cambium; the flow within each phloem bundle was unidirectional, be it up or down the stem.

In the second pattern of translocation there occurred a simultaneous (within the 25–30 min migration period) bidirectional movement of the two tracers within a single phloem bundle. Figures 2 and 4 show unmistakably that all phloem bundles within the internode, usually 17 in number, were involved to some extent in the upward transport of the C^{14} tracer. The presence of P^{32} tracer, in the course of its downward movement, was unmistakable in many of the same bundles. The conclusion is therefore inescapable that within the migration period allotted some of the phloem bundles had supported simultaneous bidirectional movement of the P^{32} and C^{14} tracers.

The resolution of the images of the phloem bundles in figures 2A and B is good, that is, the bundles are clear and distinct. This indicates that the particular portion of the bundles conducting the tracers was located very close to the cambium, i.e., it was the younger, less mature region of the phloem which was operative in conduction. It should be noted that the conduction took place in the young phloem cells rather than in the cambial cells since distinct strands, in contrast to a continuous sheath, are shown in figures 2A and B.

The simplest explanation for the bidirectional movement in the young phloem cells lying adjacent to the cambium is to invoke protoplasmic streaming for the motivating force. But this is largely an adherence to tradition, for other mechanisms involving activated diffusion and surface active forces are not excluded by our data.

The concepts of pressure flow and protoplasmic streaming (or some active mechanism), when broadly interpreted, have persisted because they conform to sound data. Recently, by a theoretical treatment, it was further shown that each mechanism could be expected to produce essentially the same distribution pattern of tracer within a stem through which it flowed (5), and that this pattern corresponded with the distribution pattern actually observed (1). The difficulty of resolving theories has never been the absence of sound data to support either one, but rather the expectation a priori that there should be but one mechanism of transport. Since there apparently are two, one showing the attributes of a physical process and the other the attributes of a metabolic process, the previous difficulty of acquiring

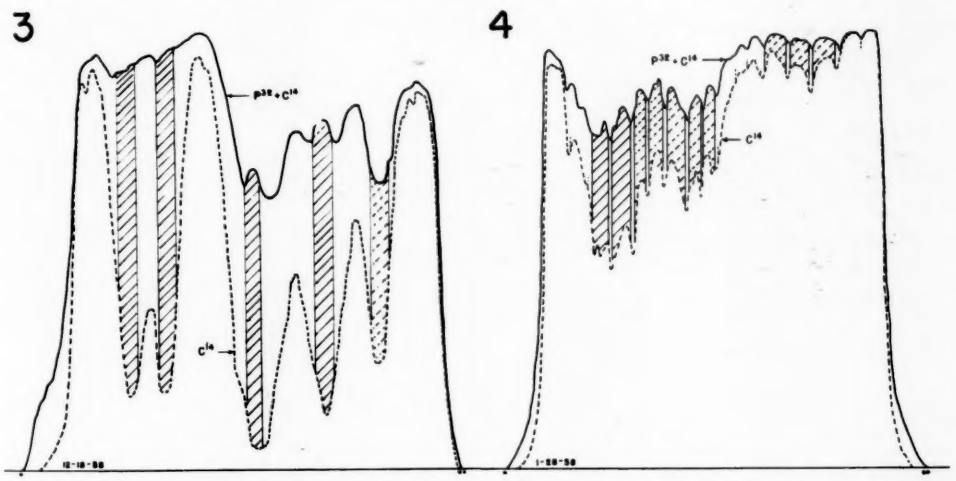

FIG. 3. Microdensitometer tracings across the radioautograms in figures 1 A and B at the position of the dots. The vertical lines with the cross hatching are located from figure 1 B with a filar micrometer and delimit the easily discernible P^{32} laden bundles. Broken cross hatching indicates lightly laden bundles. The C^{14} and P^{32} tracers move in opposite directions through separate phloem bundles. The ordinate values on the above plot are logarithmic.

FIG. 4. Microdensitometer tracings across the radioautograms in figures 2 A and B at the position of the dots. The vertical lines with the cross hatching are located from figure 2 B with a filar micrometer and delimit the easily discernible P^{32} laden bundles. Broken cross hatching indicates lightly laden bundles. The C^{14} and P^{32} tracers moved in opposite directions through many of the same phloem bundles. The ordinate values on the above plot are logarithmic.

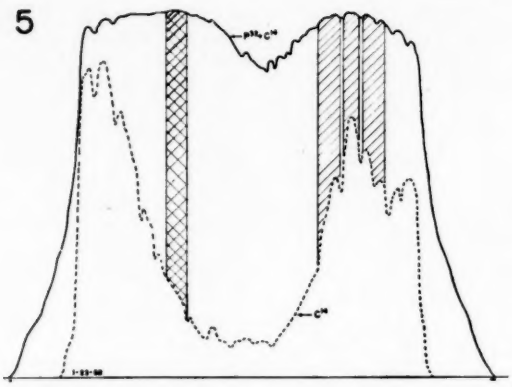

FIG. 5. Microdensitometer tracings across a radioautogram showing a combination of the two patterns of movement, such as occurred (with variations) in the majority of cases. The vertical lines with cross hatching were located from the original radioautogram with a filar micrometer and delimit the easily discernible P^{32} laden bundles. Double cross hatching indicates a heavily laden bundle. Fogging of the emulsion due to high P^{32} content of the tissue is indicated by the sloping lines near the base of the $P^{32} + C^{14}$ curve. The ordinate scale is logarithmic.

Exposure for radioautogram (not shown) A and B, 12 days 18½ hours; C, 33 days 17½ hours.

exclusively characteristic data for each can be appreciated. It should be stressed that it was the sporadic operation of each mechanism which allowed the present demonstration that the two mechanisms exist side by side.

A majority of the radioautograms showed mixed patterns of movement (fig 5), the most frequent of which appeared to be upward movement of C^{14} in the younger portions of the phloem (by protoplasmic streaming) with downward movement of P^{32} in the older portions of the phloem of the leaf traces only (by pressure flow). This is still bidirectional movement, but it differs from the two examples offered above in that the bidirectional movement involves both mechanisms. These mixed patterns were, however, rather difficult to interpret because of the degree of fogging at the edges of the bundles when the P^{32} content was appreciable. Rather than try to interpret mixed patterns from the present radioautograms, we think it more fruitful to proceed with improved techniques. We suggest finer grained film, the substitution of S^{35} for P^{32} if a 2½ year decay period can be endured, and microradioautography. We are proceeding with the latter.

The two patterns of translocation were adequately confirmed by the data obtained from the S^{35} experiments (figs 6 and 7). Here S^{35} entered the phloem primarily at the nodes; in its subsequent distribution the S^{35} assumed either one or the other of the two

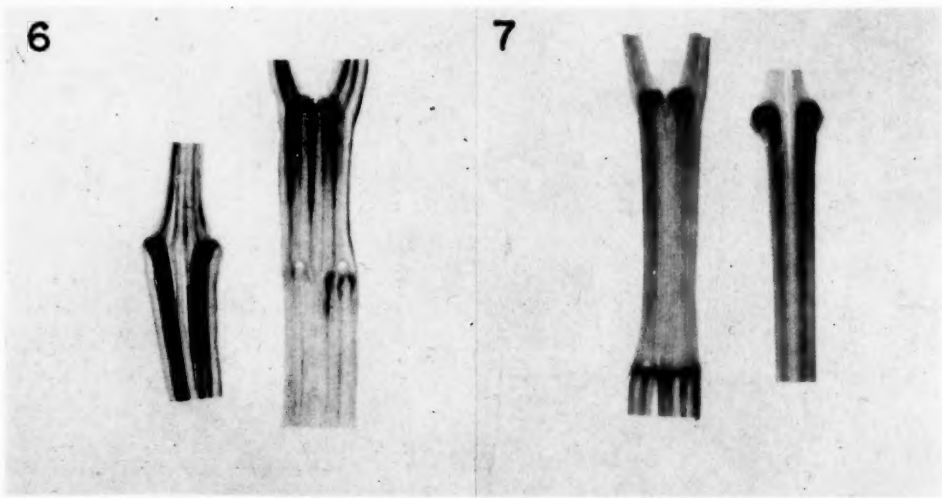

FIG. 6. A S^{35} radioautogram showing the distribution of S^{35} in the phloem bundles of a bean stem after it had entered from the xylem primarily at the nodes. Three nodes are shown with the primary leaf node at the base. The base of the section on the left joins the top of the one on the right. The angling of the stem at the nodes made severance necessary to avoid distortion of the nodal pattern. Note that the general pattern corresponds to that in figure 1A.

FIG. 7. A S^{35} radioautogram showing distribution of S^{35} in the phloem bundles of a bean stem after it had entered from the xylem primarily at the nodes. Three nodes are shown with the primary leaf node at the base. The base of the section on the right joins the top of the section on the left. The angling of the stem at the nodes made severance necessary to avoid distortion of the nodal pattern. Note that the general pattern corresponds to that in figure 2A.

distribution patterns, depending upon which distribution mechanism was in ascendancy at the moment. The first (fig 6) showed predominantly downward movement from the node in the rather deeply embedded phloem tissue of the five leaf traces. This pattern was similar to that in figure 1B and its tracing figure 3. The second (fig 7) showed a distribution, both above and below the node, in all phloem bundles which was similar to that in figure 2A. In this instance the S^{35} was present in the younger phloem cells located near to the cambial region, as attested by the fine resolution of the conducting bundles in the radioautogram. A third showed the same pattern as figure 6. Since it was possible to obtain both patterns of movement in the three trials, the sporadic occurrence of the two conducting mechanisms was confirmed.

When a movement through the symplast of young cells is proposed (as exemplified in fig 2), the question of the rate of movement immediately arises. However, from the original radioautograms (of which figs 1 and 2 are but the central portion) there was no evidence of a substantial difference in rate of movement. In both, the total distance from the place of application to the top of the stripped bark was 19 cm. This gave a known rate of upward movement of the C^{14} tracer in the phloem of 38 cm/hr; but since the concentration of the tracer was appreciable at the top of both sections, the true rate must have been considerably in excess of this value. An additional small increase is indicated because the 30-minute migration period included the time necessary to incorporate the $C^{14}O_2$ into sugar via the photosynthetic process and its export to the phloem. This process is known to proceed rapidly, however (1). All evidence indicates that the rates of movement by either process, when more accurately measured, will compare favorably with those reported earlier for downward movement of these two tracers in the phloem of the same plant. These rates are more than double the present minimal values (1).

With two mechanisms of transport in operation within the phloem, one primarily dependent upon physical forces and the other more closely allied with vital forces, the apparent conflicts which have plagued translocation data through so many years, under the view that there must be but a single mechanism of transport, would largely disappear. From the present work it seems unjustified to hope further for the simplicity that would arise if but a single mechanism were operative in translocation.

Summary

By applying $C^{14}O_2$ to the lowermost trifoliate leaf on the stem of a bean plant, and P^{32} to the next higher leaf, the simultaneous bidirectional movement of the two tracers through the phloem bundles in the included internode was studied. To do this the bark was removed, quickly frozen, and dried in vacuo. Radioautograms were prepared showing the distribution of both tracers (B), and of P^{32} alone (C), by using a filter. Then after the decay of the P^{32} a C^{14} radioautogram (A) was prepared. Radioautograms A and B were scanned with a recording microdensitometer across the same transect and by comparison (difference) the distribution of each tracer in each phloem bundle was determined.

Two distinctly different patterns of bidirectional movement were found. In the first the upward and downward movement of the tracers occurred in separate phloem bundles. In this instance the translocation appeared to occur primarily in the older more mature portions of the phloem bundles. In the second pattern the two tracers moved in opposite directions through the same phloem bundles. The younger phloem cells lying adjacent to the cambium were involved in this type of movement. The rate of upward movement of the C^{14} tracer in the phloem considerably exceeded 38 cm/hr in both patterns of movement.

The two patterns were sufficiently distinctive to require production by different mechanisms. The first pattern fits the characteristics of a pressure flow mechanism ideally; the second pattern requires an activated mechanism such as protoplasmic streaming. Because of the sporadic operation of each mechanism, examples of each pattern in pure form were found among ten trials; but combinations of the two patterns of movement were shown in the majority of cases. Since two mechanisms are indicated for the movement of substances within the phloem of the bean plant, the apparent conflicts which have plagued translocation data for so many years seem resolved and the data supporting both mechanisms appear equally valid. It seems unjustified to hope further for the simplicity that would exist if but a single mechanism were operative in translocation.

Literature Cited

1. Biddulph, O. and R. Cory. 1957. An analysis of translocation in the phloem of the bean plant using THO, P-32 and $C^{14}O_2$. Plant Physiol. 32: 608–619.
2. Biddulph, O., R. Cory, and Susann Biddulph. 1959. Translocation of calcium in the bean plant. Plant Physiol. 34: 512–519.
3. Biddulph, O., and Jane Markle. 1944. Translocation of radiophosphorus in the phloem of the cotton plant. Amer. Jour. Bot. 31: 65–70.
4. Chen, S. L. 1951. Simultaneous movement of phosphorus-32 and carbon-14 in opposite directions in phloem tissue. Amer. Jour. Bot. 38: 203–211.
5. Horwitz, L. 1958. Some simplified mathematical treatments of translocation in plants. Plant Physiol. 33: 81–93.
6. Rabideau, G. S., and G. O. Burr. 1945. The use of the C^{13} isotope as a tracer for transport studies in plants. Amer. Jour. Bot. 32: 349–356.

CONSTRUCTION OF LARGE LOW COST FILTERS FOR PLANT GROWTH STUDIES[1,2]

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Many problems in biology require the isolation of fairly narrow spectral regions so that the effect of radiation of various wave-lengths may be determined. When fairly large areas need to be illuminated at high intensities the use of commercially available filters or monochromators is not practical. Therefore, light filters made up of selectively absorbing materials must be used. This report describes the construction of a series of filters which we needed in our study of the effects of light quality on plant morphogenesis.

In an attempt to isolate and define the limits of anti-rachitic radiations Hess and Weinstock (1) used light filters made of selectively absorbing materials. A detailed description of a similar set of filters was given by Jones (2) in 1929. Withrow and Price (6) published details on the preparation of dyed gelatin films and presented data on the transmission of water and of salt solutions which are useful as primary filters for removing infrared radiation. A procedure for preparing dyed plastic filters was presented by Shenk et al (4), and the transmittancies of some commercially available plastics have been given by King and Ventura (3).

In order to study the development of higher plants in light of narrow spectral regions Wassink and Stolwijk (5) employed combinations of selected light sources and colored glass filters to illuminate specially constructed growth cabinets.

The spectral transmission and cost of commercially available light sources and filters were investigated by the authors. Based on this information and on various reports from the literature it was decided to fabricate the filters, reported upon herein, from commercially available plastics.

MATERIALS AND METHODS

Samples of various colored plastics [sold under the trade names of "Perspex" and "Plexiglass" (polymethylmethacrylate)] were obtained, and the transmission spectra of these were determined with a Beckman DK-1 spectrophotometer. These data were employed in deriving the combinations of plastics and concentrations of copper sulphate required for various filters.

The rather high transmittance in the red and infrared regions by all the plastics made it necessary to fabricate the filters as completely enclosed tanks to contain aqueous copper sulphate, which effectively absorbs these bands.

The plastics were cut to the desired dimensions and were fabricated as enclosed tanks using 1,2-dichloroethane as the binding solvent. The control filter was made of clear plastic and filled with dis-

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[2] This work was supported by the National Research Council of Canada and by a University of Alberta Research Grant.

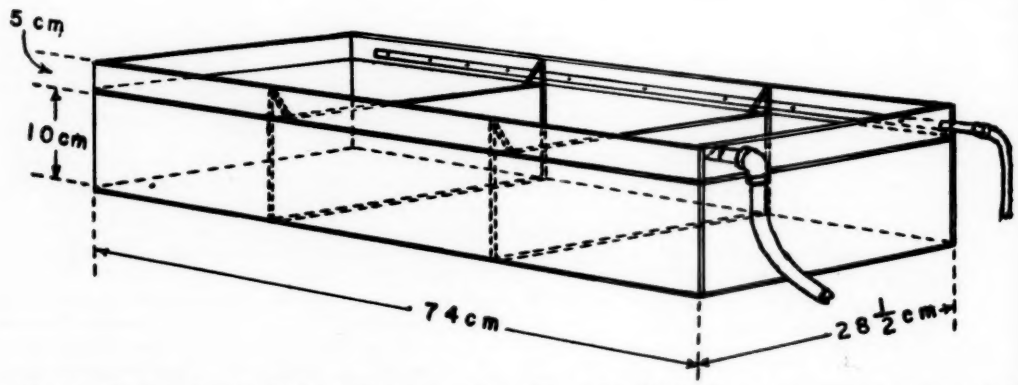

FIG. 1. Diagram showing detail of filter construction. The tank is filled with water or aqueous copper sulphate. The 5 cm enclosure above the tank is for coolant. A perforated plastic tube extending along the length of the filter serves as the water inlet. The coolant water is held to a depth of 3 cm by the set of the drain and adjustment of the rate of water flow.

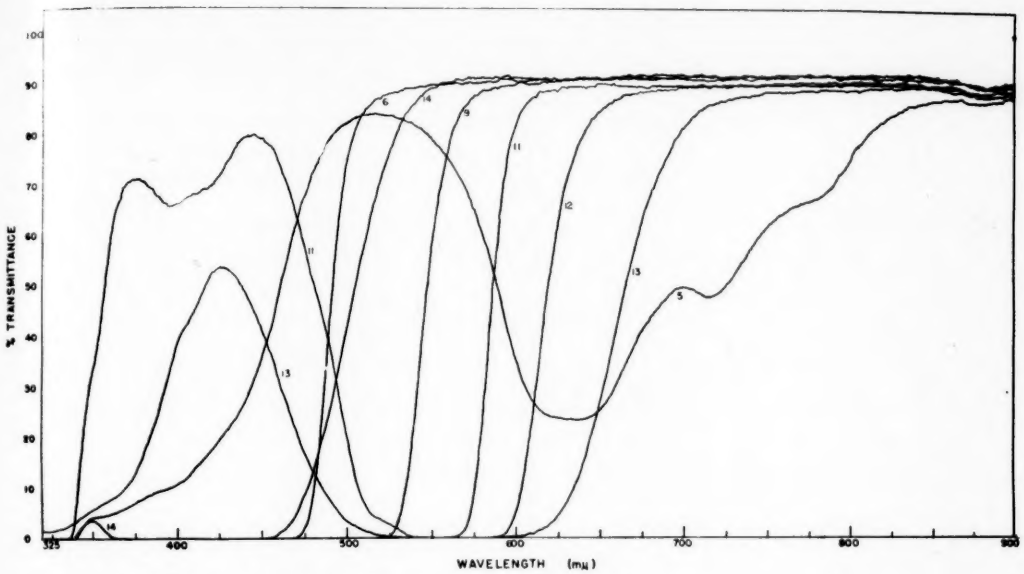

FIG. 2. Transmittance of colored plastics used in preparing filters.

tilled water. Colored plastics were used individually or in combination in the other eight filters, which were filled with the required concentration of aqueous copper sulphate.

The filters fit on light-tight growth cabinets; light assemblies of incandescent lamps are placed above the filters. In view of the high heat irradiance of incandescent lamps the filters must be protected from high temperatures. Therefore, a 5 cm enclosure around the top of the filter was fitted with an inlet and drain to allow constant flow of a 3 cm layer of tap water over the surface of the filter.

DESCRIPTION OF FILTERS

A diagram of a complete filter is shown in figure 1. The advantage of the all-plastic construction is apparent. The tank design makes it possible to combine the aqueous and plastic filters into a sturdy compact unit. In addition, the enclosure around the top of the filters permits direct use of water as a coolant.

The transmittance of the following plastics is shown in figures 2 and 3. All colored plastics used were ⅛ inch in thickness.

TABLE I

COMPOSITION AND TRANSMITTANCE DATA ON FILTERS

FILTER NO.	PLASTIC	COPPER SULPHATE* GM/L	WAVELENGTH MμAT PEAK–50 % TRANSMISSION	
1	13 + 15	50	426	393–463
2	11 + 5	150	465	436–492
3	5 + 5	75	493	460–532
4	6 + 14	20	532	500–577
5	9 + 15	10	560	540–605
6	9 + 11	3	600	580–655
7	12 + 15	1.5	636	610–705
8	9 + 13	1.5	680	646–738
9	15 + 15	Water		346–890

* All solutions made up in 0.5 % sulphuric acid.

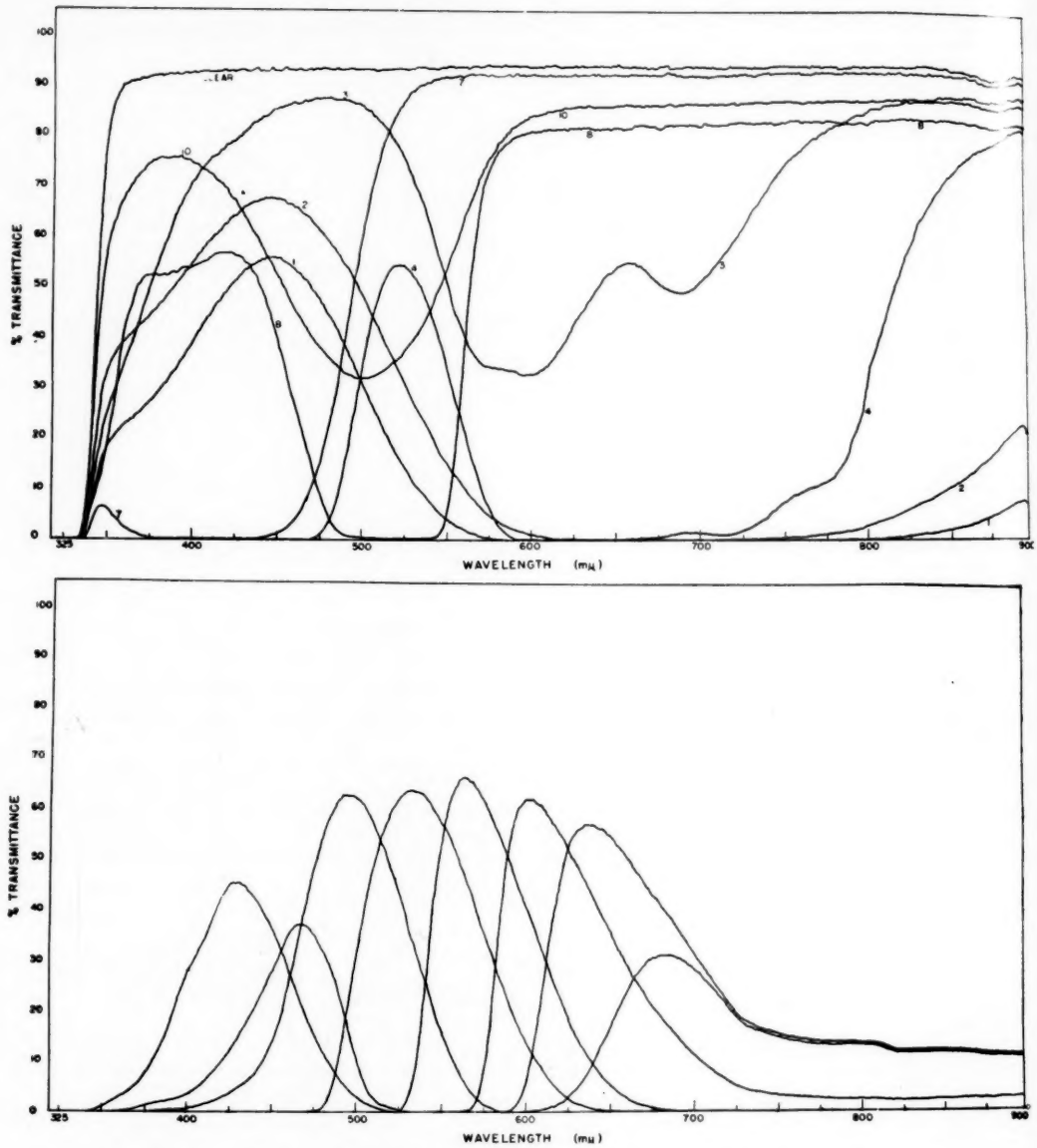

FIG. 3. Transmittance of clear plastic and of colored plastics that were tested but not used.

FIG. 4. Transmittance of set of filters described in table I. The per cent transmittance for a path length of 10 cm of the copper sulphate concentration used plus the appropriate plastics balanced against air path.

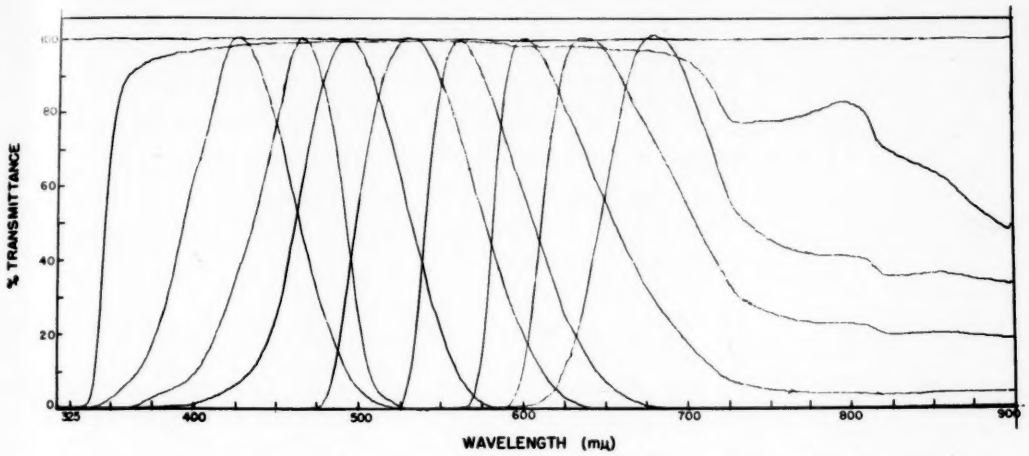

FIG. 5. Transmittance of set of filters described in table I with all peaks adjusted to 100 % transmittance.

1. Blue 2045[3]
2. Blue 2083
3. Blue 2069
4. Green 2092
5. Green 2082
6. Green 2154
7. Yellow 2048
8. Yellow 2086
9. Amber 2451
10. Red 2081
11. Red 2085
12. Red 2444
13. Blue 705
14. Yellow 2208
15. Clear

The plastics represented in figure 2 and the clear plastics were used in building the filters. Employing these data on transmittance and the information given by Withrow and Price (6) on the transmittance of copper sulphate solutions it was possible to derive the combinations of plastics and copper sulphate solutions reported in table I. It shows the wavelength peaks of the various filters and their wavelengths at 50 % transmission.

The per cent transmittance for the complete set of filters is given in figure 4. Although the per cent transmittancies of the filters varies this will in practice be corrected by adjusting the incident light intensities to provide uniform energy below all the filters. Figure 5 shows the transmittance of the set of filters with the peaks adjusted to 100 % transmittance.

[3] Manufacturers' color identifications. Number 13 is Perspex (Imperial Chemical Industries, Ltd), the others Plexiglass (Rohm and Haas Co.).

SUMMARY

A set of filters for isolating narrow spectral regions is described. They are made as tanks to contain various concentrations of copper sulphate solution which together with the colored plastics provide a series of filters that are useful for photobiological studies.

LITERATURE CITED

1. HESS, A. F. and MILDRED WEINSTOCK. 1923. A study of light waves in their relation to rickets. Jour. Amer. Med. Assoc. 80: 687–690.
2. JONES, L. A. 1929. Isolation of narrow spectral regions by selective absorption. Jour. Nutrition 2: 111–123.
3. KING, E. J. and S. VENTURA. 1951. Light filters of colored "Perspex". Nature 168: 702.
4. SHENK, J. H., E. S. HODGE, R. J. MORRIS, E. E. PICKETT, and W. R. BRODE. 1946. Plastic filters for the visible and near infra-red regions. Jour. Opt. Soc. Amer. 36: 569–575.
5. WASSINK, E. C. and J. A. J. STOLWIJK. 1952. Effects of light of narrow spectral regions on growth and development of plants. Proc. Koninkl. Ned. Akad. C 55: 471–488.
6. WITHROW, R. B. and L. PRICE. 1953. Filters for the isolation of narrow regions in the visible and near visible spectrum. Plant Physiol. 28: 105–114.

NITRATE REDUCTASE ACTIVITY IN CORN SEEDLINGS AS AFFECTED BY LIGHT AND NITRATE CONTENT OF NUTRIENT MEDIA[1, 2]

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The requirement of light for the reduction of nitrate by higher plants has been under investigation for some time. Burstrom (2) obtained evidence that wheat leaves would reduce nitrate in the light but not in the dark. He concluded that nitrate reduction was directly linked to a photochemical reaction, or that it occurred concomitantly with the fixation and reduction of carbon dioxide. In contrast, Delwiche (5) used isotopic nitrogen to demonstrate that tobacco plants metabolize nitrate or ammonia in the dark as well as in the light. Other workers (7, 13) have demonstrated that nitrate and hyponitrite are metabolized both in dark and light but that in light the rate is accelerated.

The extraction, purification, and characterization of a pyridine nucleotide-nitrate reductase from soybean leaves and the occurrence of this enzyme in other higher plant species was reported in 1953 (6). Other aspects of nitrate reductase metabolism and characteristics have been published by Nason (14), Nicholas and Stevens (15), and Cheniae and Evans (4).

In 1956 experiments were initiated on the Agronomy South Farm, Urbana, to determine the effect of shade, both artificial and self-(competitive plant) shading on the nitrate metabolism of corn plants. It was concluded from these experiments (12) that nitrate accumulated in plants grown under conditions of low light intensity and that nitrate metabolism in two lines of corn (Hy2 × Oh7 and WF9 × C103) were differentially affected by the shade treatments. Candella et al (3) demonstrated that cauliflower plants slowly lost their nitrate reductase activity when placed in the dark. The nitrate reductase activity returned rapidly when the plants were returned to light.

The experiments described in this report were carried out to determine effects of light and nitrate supply on the activity of nitrate reductase in young corn plants.

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[2] This investigation was supported by National Science Foundation Grant No. 4407 and by Federal funds granted to the Illinois Agricultural Experiment Station, University of Illinois, Urbana.

MATERIALS AND METHODS

PLANT MATERIAL: The shoots of corn (*Zea mays* L.) seedlings or the deribbed leaves of older plants of the two hybrids (WF9 × C103 and Hy2 × Oh7)[3] were used as source material. Shoot and leaf tissue were used because the level of nitrate reductase activity was lower (80 %) in the root extracts. WF9 × C103 seeds were larger (0.31 g/seed) than Hy2 × Oh7 (0.23 g/seed) and normally produced a larger more vigorous seedling and vegetative plant. These two lines were selected because their agronomic characteristics were known (12).

SAMPLING AND EXTRACTION OF ENZYMES: The shoot portion or leaves were removed from four to six plants and composited to form a sample from each treatment. The sample was immersed immediately in cold (2° C) deionized water and carried to the laboratory. The shoots or deribbed leaves were blotted dry, weighed, cut into small pieces, and ground in an Omnimixer at maximum speed for 2 minutes. The grinding medium was 0.1 M Tris,[4] 0.01 M cysteine, and 0.0003 M EDTA at a pH of between 7.3 and 7.8 (adjusted with HCl). The precise pH was determined experimentally for each kind of tissue so that the homogenate would have a pH of approximately 7.0 after grinding. Four ml of cold (2° C) grinding medium were added for each gram of tissue. The homogenate was pressed through cheesecloth and centrifuged[5] for 15 minutes at 20,000 G. The supernatant liquid was decanted through glass wool and assayed. The homogenates and extracts were kept cold (3–5° C) throughout. The assays were completed within 2 to 3 hours after sampling.

[3] The seeds were obtained from the Illinois Seed Producers Association through the courtesy of Mr. F. S. Ingersoll. The seeds were selected for uniformity of size and appearance.

[4] The following abbreviations are used: Tris for tris (hydroxymethyl) aminomethane; EDTA for ethylenediaminetetraacetic acid; TPD, triosephosphate dehydrogenase; and DPN and TPN for di- and tri-phosphopyridine nucleotides, respectively.

[5] All centrifugations were carried out in refrigerated centrifuges.

ASSAYS: The nitrate reductase was measured by a modification of the method described by Evans and Nason (6). The assay mixture contained 1.0 ml of 0.1 M potassium phosphate buffer, 0.2 ml of 0.1 M KNO_3, 0.5 ml of 1.36×10^{-3} M DPNH, 0.2 ml of enzyme extract, and deionized water to bring volume to 2.0 ml. The assay was initiated by addition of, first, DPNH and immediately thereafter the enzyme extract. The mixture was incubated at 27° C for 15 minutes and the reaction stopped by adding 1 ml of 1 % w/v sulfanilamide in 1.5 N HCl. N-(1 napthyl) ethylene diamine hydrochloride reagent was added (1 ml of 0.02 % w/v) and the contents mixed by inverting the tubes. The color was allowed to develop for 5 minutes before centrifuging at 1,500 G for 10 minutes to remove the turbidity. The absorbancy was determined by reading each sample against its own blank (complete except for DPNH) in a Beckman DU spectrophotometer at 540 mμ. Nitrate reductase extracted from corn, like that extracted from germinating wheat (16) is DPN-specific. Adding flavin nucleotide to the assay mixture did not enhance the activity and therefore it was not used. Enzyme extracts that were heated for 3 minutes in boiling water were inactive. The production of nitrite was linear with enzyme extract added over the range 0.05 to 0.6 ml. Hydroxylamine hydrochloride at the concentration suggested by Evans and Nason (6) was not used because it inhibited the activity of the enzyme. Nitrite which was added as potassium nitrite was lost from supplemental blanks that were complete except for DPNH. Although this loss of nitrite was relatively constant and non-enzymatic, it was not used as a correction factor. The activity of the nitrate reductase was expressed as μm KNO_2 formed per hour per gram fresh weight or per shoot.

Triosephosphate dehydrogenase activities were determined by a procedure previously described (9). The DPN-dependent TPD was selected as a representative enzyme, to determine if prolonged darkness inactivates enzymes in general. The TPN-dependent TPD was selected because it has been shown (8) to possess certain light-dependent characteristics.

Nitrate content was estimated by the method of Wooley et al (18). The protein content of the extracts was determined by Nesslerizing the 5 % trichloroacetic acid precipitable material (17). Bovine serum albumin was used for the reference protein.

Single determinations were made on each sample. Replication was obtained by repeating the experiment with separate lots of material.

CULTURAL TECHNIQUES AND EXPERIMENTAL PROCEDURES: *Experiment I.* The plants were grown in fertile soil in 6-inch clay pots under normal greenhouse conditions. Ten seeds were planted per pot and the plants thinned to five within 10 days. Adequate soil moisture was maintained with deionized water. Dark treatments were initiated when the plants were 21 days old, by transferring the plants to a darkened basement and placing the pots under a large (2′ × 2′ × 3′) box. Compressed air was bled into the box at the rate of 5 l/min to prevent excessive accumulation of CO_2 and moisture. The temperature varied from 24 to 28° C. A 5-watt red light bulb provided light at sampling time, otherwise the plants were in complete darkness.

Experiment II. Cultural techniques were the same as used in experiment I.

Variation in light intensity was obtained by placing the plants under box frames (30″ × 60″ × 30″) that were covered with Saran screen of varying mesh. The percentage of light transmitted was measured with a Weston (Model 603) light meter. When this series of experiments was conducted the greenhouse was covered with whitewash. This coating reduced the light intensity incident at the top of the shade structures and the unshaded plants to 6,000 to 7,000 ft-c on clear days at noon. The two shade treatments, which further reduced the light intensity 25 and 90 %, were applied 6 days after the seeds were planted.

Experiment III. Seedling material used in this experiment was grown in 250 g Terra-Lite a commercial expanded vermiculite in 3-quart Pyrex utility dishes. Forty seeds were planted in each dish. The vermiculite was wetted at planting time with 10^{-4} M $CaCl_2$ or No. 1 Hoagland solution (11) at the rate of 470 ml/100 g vermiculite. Deionized water or nutrient solution was added as needed to maintain the initial moisture level.

The material was grown in a controlled environmental chamber regulated at 22 to 23° C and 65 to 75 % relative humidity. Light was supplied by an overhead bank of 30 cool white, 72-inch fluorescent tubes and supplemented with twelve 60-watt incandescent bulbs. Incident light intensity (measured with a Weston light meter) at the top of the plants was approximately 1,750 ft-c.

Three sets of treatments were used. In set one, the seedlings were supplied with a No. 1 Hoagland solution and were germinated and grown in complete darkness. Darkness was obtained by covering with an aerated but light tight box.

A second set of seedlings was handled in an identical manner until the seedlings were 8 days old. At this time they were transferred into the light (1,750 ft-c, continuous). A third set of seeds was watered with a modified Hoagland solution that contained no nitrate (equimolar amounts of sulfate salts were substituted) and germinated in the light (1,750 ft-c—16 hr day). After the 8th day this third set was illuminated, continuously, (1,750 ft-c).

The plants were harvested and assayed at the initiation of illumination and after 5 and 26 hours of continuous illumination for sets two and three. Plants in set one (continuous dark) were harvested at the same time intervals to obtain material of comparable age.

Experiment IV. The cultural techniques were the same as in experiment III.

The following treatments were used. Half of the material was supplied with No. 1 Hoagland solution and the remainder with a modified "no nitrogen"

Hoagland solution. All plants were germinated in the dark for 8 days. On the 8th day all plants were transferred into the light and at the same time 12.5 mm of nitrate were added to each utility dish that held "no nitrate" plants. This was the same amount of nitrate that had been supplied initially in the complete Hoagland media.

Experiment V. Cultural techniques were the same as in experiment I, except that quartz sand and Hoagland No. 1 nutrient solution, with micro-nutrient supplement, were used for the nutrient medium. Excess solution was added at each watering and permitted to drain from the pots. The pots were flushed with tap water periodically to prevent excess salt accumulation. Equimolar quantities of K_2SO_4 or $CaSO_4$ were substituted for KNO_3 and $Ca(NO_3)_2$ in the nutrient solutions to obtain the differential nitrogen treatments.

Experimental Results

Experiment I. This experiment was conducted to determine the effect of complete darkness on the activity of nitrate reductase and triosephosphate dehydrogenases and the water soluble protein content of young corn.

The results are shown in figure 1 (A, B, C and D). The initial level of activity of the enzymes and protein content was established by sampling on 2 successive days prior to the dark treatment. The nitrate reductase dropped to less than 10 % of the original level of activity in both hybrids within 48 hours after the plants were placed in the dark. Nitrate reductase was not detectable in the extracts prepared from the WF9 × C103 seedlings after 96 hours of darkness and was present only in trace amounts in extracts from Hy2 × Oh7 material.

An increase in nitrate reductase was detectable within 2 hours after the plants were returned to full sunlight in the greenhouse after 65 hours of darkness. Thirty hours after removal of the plants from the dark the activity of nitrate reductase had returned to 60 % of the initial level of activity. Approximately one-third of this 30-hour period was in darkness (night). Some leaves on the plants were damaged after the plants were transferred back to the light. The damaged leaves were removed from the plants

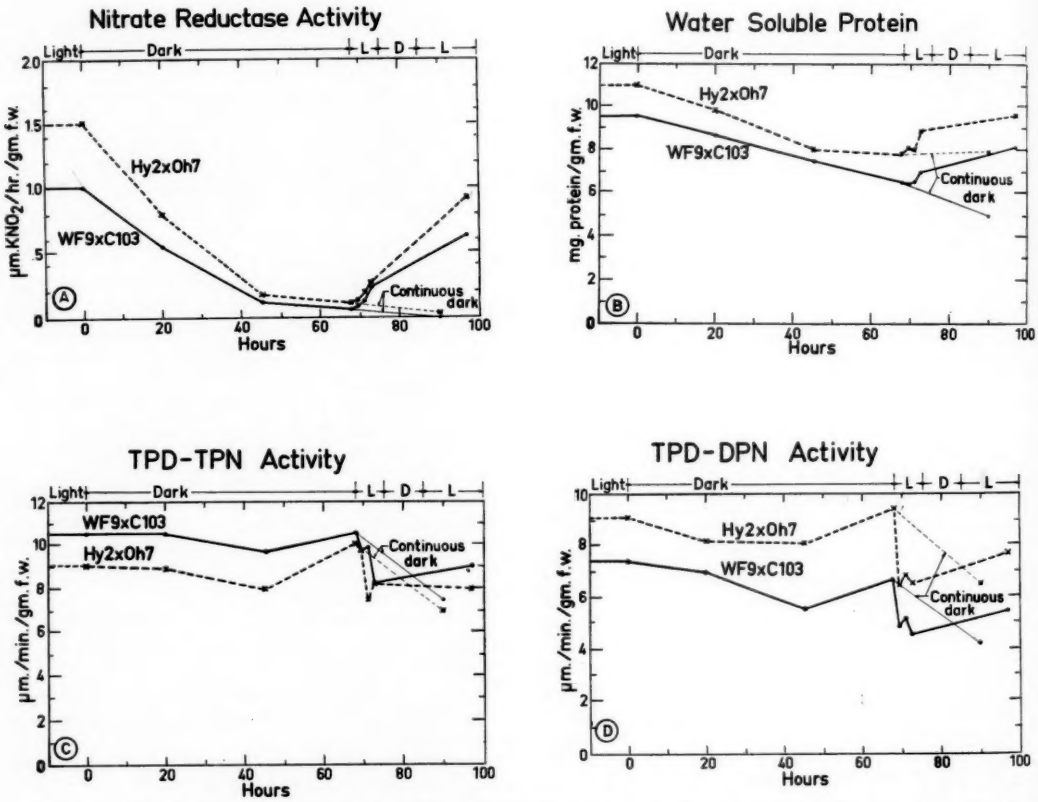

Fig. 1. Effect of dark treatment on the nitrate reductase (A) and triosephosphate dehydrogenases activities TPN (C) and DPN (D) and the protein content (B) of young corn plants.

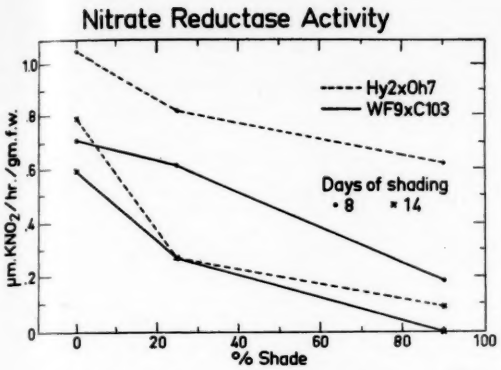

FIG. 2. Effect of shading on the nitrate reductase activity of young corn plants. (Assays made on leaf extracts).

and discarded. Plants that were transferred to the light after 96 hours of darkness did not survive.

Water-soluble protein content of the plants decreased during the dark treatment and increased when the plants were returned to the light as shown in figure 1-B. The changes in protein content of the plant correlated positively with the changes in nitrate reductase activity [fig 1 (A, B)] obtained with the light and dark treatments.

The DPN- and TPN-dependent triosephosphate dehydrogenases were not affected to any great extent by the alternate treatments of light and dark [fig 1 (C, D)]. The TPN-dependent TPD activity was consistently higher in the WF9 × C103 hybrid than in the Hy2 × Oh7 hybrid, while the converse was observed for the DPN-dependent TPD. Additional work is contemplated to determine if these are genetic characteristics of these hybrids. The TPD activity per unit of protein with both coenzymes increased throughout the first 72 hours of darkness but decreased during the next 24 hours. The increase in specific activity during the first 72 hours is attributed to the decrease in total protein with no accompanying decrease in TPD.

Experiment II. The effect of various levels of shade and duration of shade treatment on nitrate reductase activity, growth, and water soluble protein content of corn seedlings was determined. Figure 2 shows that the nitrate reductase activity decreased roughly in proportion to the amount of shading. Prolonging the shade treatment caused further decrease in enzymatic activity. After 14 days little or no nitrate reductase could be detected in either the Hy2 × Oh7 or WF9 × C103 plants grown under the 90 % shade structure.

TABLE I

EFFECT OF SHADING ON FRESH WEIGHT AND PROTEIN CONTENT OF YOUNG CORN SEEDLINGS

TREATMENTS % SHADE	FRESH WT G/SHOOT*		PROTEIN MG/SHOOT**	
	DAYS SHADED			
	8 DAYS	14 DAYS	8 DAYS	14 DAYS
	Hy2 × Oh7			
0	1.98	6.00	17.2	44.5
25	1.54	5.00	10.9	30.5
90	1.06	1.90	11.5	8.4
	WF9 × C103			
0	3.86	9.50	29.7	77.0
25	2.44	6.50	21.7	42.0
90	1.65	2.50	7.6	11.5

* Total portion of plant above ground level.
** Leaves only. The same as used for the nitrate reductase activity.

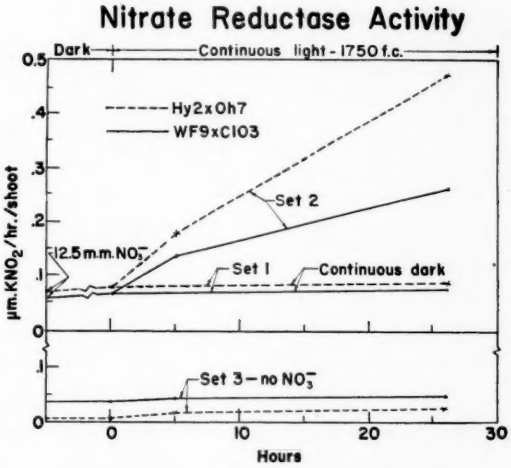

FIG. 3. Nitrate reductase activity of young corn seedlings grown under various light and dark treatments with and without nitrate. Set 1. Seedlings grown in continuous dark with 12.5 mm nitrate per dish. Set 2. Seedlings grown same as set 1 except that they were transferred to continuous light (1,750 ft-c) when 192 hours old. Set 3. Seedling grown in light (1,750 ft-c-16-hour day) without nitrate and transferred to continuous light (1,750 ft-c) when 192 hours old.

As shown in table I, growth (fresh wt) of the plants was also reduced, roughly in proportion to the amount of shading. The protein content per shoot (table I) was affected in a similar manner, especially after 14 days of shade treatment. There is a positive correlation between the nitrate reductase (fig 2) and the protein content and growth (table I) of these plants.

Experiment III. Figure 3 shows the effect of continuous dark and light and dark→light treatments on nitrate reductase activity in corn seedlings grown

TABLE II

COMPARISON OF FRESH WEIGHT AND WATER SOLUBLE-PROTEIN CONTENT OF YOUNG CORN SEEDLINGS GROWN UNDER VARIOUS LIGHT AND DARK TREATMENTS WITH AND WITHOUT NITRATE

TREATMENT	AGE (HRS)	FRESH WT G/SHOOT*			PROTEIN MG/SHOOT*		
		192	197	218	192	197	218
			Hy2 × Oh7				
Set 1** + NO_3^- Dark		0.52	0.60	0.79	2.8	2.9	3.2
Set 2*** + NO_3^- Dark→Light		0.51	0.63	0.78	2.9	2.9	4.5
Set 3† − NO_3^- Light→Light		0.44	0.48	0.50	2.1	2.2	2.6
			WF9 × C103				
Set 1** + NO_3^- Dark		0.62	0.74	0.82	3.7	3.8	3.8
Set 2*** + NO_3^- Dark→Light		0.63	0.69	0.84	3.8	3.8	6.2
Set 3† − NO_3^- Light→Light		0.62	0.64	0.62	3.4	4.3	3.9

* Total portion of plant above ground.
** Seedlings grown in continuous dark with 12.5 mm nitrate per dish.
*** Seedlings grown same as set 1 except transferred to continuous light (1,750 ft-c) when 192 hours old.
† Seedlings grown in light (1,750 ft-c—16-hour day) without nitrate and transferred to continuous light (1,750 ft-c) when 192 hours old.

TABLE III

EFFECTS OF LIGHT AND NITRATE ADDITION TIME ON GROWTH AND PROTEIN CONTENT OF 2 CORN HYBRIDS

LIGHT TREATMENT	FRESH WT G/SHOOT*		PROTEIN MG/SHOOT*	
	$+NO_3$**	−, $+NO_3$***	$+NO_3$**	−, $+NO_3$***
		Hy2 × Oh7		
Dark—8 days	0.75	0.59	3.1	2.6
Light—hours				
4	0.73	0.60	3.1	2.3
8	0.77	0.61	3.2	2.4
28	0.92	0.67	5.2	3.6
55	0.90	0.70	5.9	4.2
		WF9 × C103		
Dark—8 days	0.89	0.68	4.2	3.4
Light—hours				
4	0.90	0.70	4.6	3.1
8	0.95	0.70	5.1	3.5
28	0.98	0.75	6.2	4.7
55	1.26	0.88	9.2	6.8

* Total portion of plant above ground level.
** Plants were germinated in vermiculite wetted with Hoagland solution. Deionized water was added as needed thereafter.
*** Plants were germinated in vermiculite wetted with a modified Hoagland solution devoid of nitrogen. When the plants were transferred into the light, 12.5 mm of nitrate was added to each dish. Deionized water was added as needed, throughout.

with and without nitrate. Both nitrate and light are necessary for the formation of nitrate reductase in quantities required by plants for normal growth.

Other experiments have shown that plants grown without nitrate in the greenhouse under normal light cycles have a level of nitrate reductase activity comparable to that shown for the "no-NO_3^- plants" (fig 3), however after 7 or 8 days even this low level of nitrate reductase activity could not be detected.

The data show that the dark-grown seedlings are capable of reducing nitrate at a slow rate (0.06 to 0.07 μm KNO_3/hr/shoot). This suggests that the light effect is indirect. Fresh weight and water soluble protein content of these plants are given in table II.

There was a positive correlation between the nitrate reductase activity and protein content of the plants. A comparison of the protein content (table II) with the nitrate reductase activity (fig 3), suggests that a portion of the increase in nitrate reductase activity was the result of synthesis.

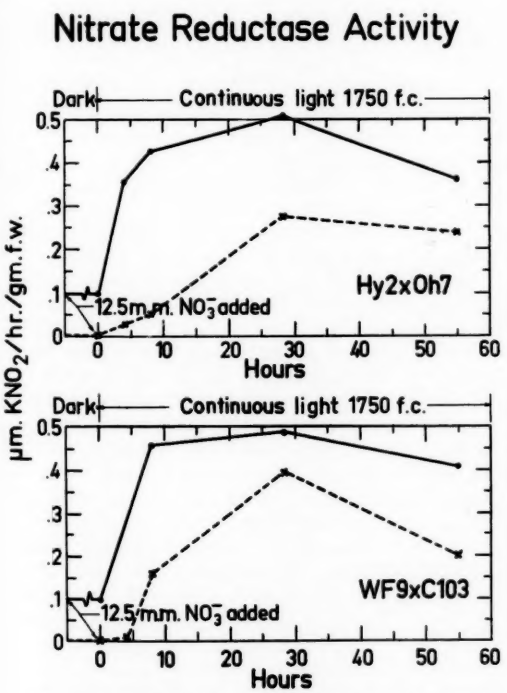

FIG. 4. Effect of light and time of nitrate addition on the nitrate reductase activity of young corn shoots. Solid lines indicate the nitrate reductase activity in seedlings germinated in the dark with nitrate (Hoagland solution) and transferred into the light at zero time. Dotted lines indicate the nitrate reductase activity in seedlings germinated in the dark without nitrate (modified Hoagland solution) but supplied with nitrate at the time of transfer into the light.

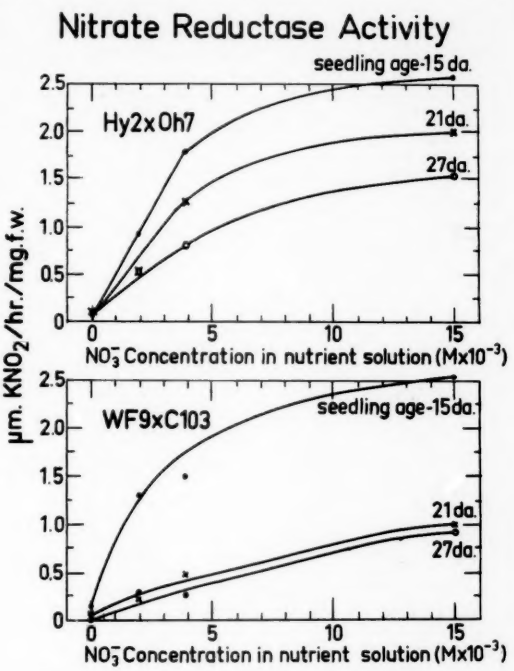

FIG. 5. Nitrate reductase activity of young corn shoots grown in media that contained various concentrations of nitrate.

Experiment IV. Studies were made concerning the rate of synthesis of nitrate reductase in corn seedlings upon exposure to light. Typical results are presented in figure 4 and table III. The hybrid WF9 × C103 formed nitrate reductase more rapidly than hybrid Hy2 × Oh7 (fig 4) when the nitrate was added at the same time the plants were placed in the light. This response is attributed to the more vigorous seedling growth, and ability to accumulate ions that is characteristic of WF9 × C103.

Experiment V. Nitrate reductase activity was increased in corn seedlings as the concentration of nitrate was increased in the nutrient solution. The results of one set of treatments are shown in figure 5.

In general, there was a positive correlation between the growth (fresh wt) or protein content of the plants (table IV) and the nitrate reductase activity (fig 5). The WF9 × C103 plants grown in the medium supplied with 0.0038 M nitrate were chlorotic and stunted. Although no reason is known for the aberrant growth, the effect was reflected in the fresh weight, protein and nitrate content of these plants (table IV).

Similar results were obtained with plants grown in vermiculite cultures and supplied with varied concentrations of nitrate.

TABLE IV

GROWTH, PROTEIN, AND NITRATE CONTENT OF YOUNG CORN SHOOTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITRATE IN NUTRIENT MEDIA

SEEDLING AGE DAYS	Hv2 × Oh7			WF9 × C103		
	15	21	27	15	21	27
	Fresh wt (g/shoot)*					
Conc. NO_3^- in media M						
0.0	0.35	0.51	0.67	0.75	1.17	2.61
0.0019	0.40	1.28	2.03	0.95	2.40	5.31
0.0038	0.53	1.35	2.40	0.84	1.56	3.15
0.015	0.56	1.50	3.07	0.90	2.41	5.60
	Protein (mg/shoot)					
0.0	1.7	1.6	2.2	4.5	4.2	4.8
0.0019	3.4	6.3	12.6	8.3	10.8	25.5
0.0038	4.7	9.8	16.1	7.2	10.9	14.8
0.015	5.5	13.4	20.6	8.6	17.4	35.3
	NO_3^- N (ppm/g ft wt)					
0.0	N.D.**	N.D.	N.D.	N.D.	N.D.	N.D.
0.0019	N.D.	N.D.	25	310	tr	140
0.0038	340	250	270	310	15	75
0.015	1,240	740	1,200	1,580	730	1,540

* Total portion of plant above ground level.
** Not detectable.

DISCUSSION

At present no explanation can be given for the loss of nitrate reductase activity in the plants placed in the dark (Expt I). The loss of protein (fig 1–B) and the accumulation of nitrate (data to be presented elsewhere) when the plants are placed in the dark, argues that this loss of enzyme activity occurs in vivo and is not an artifact of isolation. Two possible causal factors are the accumulation in the dark of an inhibitory nitrogenous metabolite and the oxidation of the active sulfhydryl group(s) (6) on the enzyme. Some information has been obtained on the former. Plant material grown in the dark was mixed with an equal portion of material exposed to normal greenhouse light prior to grinding. Assays made on extracts prepared in this manner did not indicate the presence of an inhibitor in the dark-grown material of either Hy2 × Oh7 or WF9 × C103. Similar results were obtained by mixing of extracts prepared separately from the light- and dark-grown materials. Adding hydroxylamine to the assay in final concentrations of 10^{-4} and 10^{-3} M caused an inhibition of nitrate reductase activity of 25 and 78 %, respectively. It is doubtful if hydroxylamine would ever reach these concentrations in plant material as a natural metabolite. Ammonia as ammonium sulfate, asparagine, and glutamine (10^{-3} M) did not inhibit nitrate reductase activity.

Under dark conditions the oxidation-reduction systems of the plant may shift toward oxidation thereby inactivating certain enzyme systems. The return to the light should reverse this process which in turn may reduce an inactive, oxidized enzyme. Unless some specific oxidizable-reducible compound or localization of nitrate reductase in the tissue is envisaged, this postulate is untenable because of the stability of the sulfhydyl-active triosephosphate dehydrogenases under similar conditions (fig 1–C and 1–D).

No adequate explanation can be given for the decrease in nitrate reductase at the last sampling (55 hrs light, fig 4, Expt IV). The decrease in nitrate reductase with age of plants grown in vermiculite was observed repeatedly under varied environmental conditions. Although corn seedlings in vermiculite are vigorous and appear healthy up to periods of 12 to 14 days, growth thereafter is extremely slow and the plant becomes spindly. The use of greater amounts of vermiculite per plant, aeration, or modification of nutrient supply did not change this pattern of growth.

In experiment V, the loss of nitrate reductase activity with seedling age is attributed, in part, to the proportionate increase in stem tissue. The expanded leaves have the highest level of nitrate reductase. In these assays the entire shoot portion was homogenized, thereby increasing the stem to leaf ratio.

Other environmental factors, such as excessively high salt content in the tissue for prolonged periods, may also reduce nitrate reductase activity by some unknown mechanism. The nitrate concentration in the tissue grown in Hoagland solution (0.015 M nitrate) (table IV) is well in excess of 0.02 M that has been reported by Evans and Nason (6) to be inhibitory

(75%) to the photosynthetic reduction of TPN. Since these plants were not subdivided into leaf and stalk portion, the relative nitrate content in the individual parts is not known. In general, the stalk portion of corn plants is higher (5–20-fold) in nitrate content than the leaves (10). However, these high concentrations of nitrate (table IV) did not suppress growth and protein synthesis in comparison with the effects obtained with media containing lower levels of nitrate. This does not preclude the possibility that uniform application of intermediate levels of nitrate-containing media might produce larger plants.

In general, agronomists have observed that corn plants accumulate nitrate (tissue tests) under various conditions of environmental stress. Conditions of droughty or water-logged soil, reduced light or darkness, as well as deficiencies in phosphate and iron, have been associated with high nitrate accumulation in corn leaves (Pers. communic. from Prof. Edward H. Tyner, Agronomy Dept., Univ. of Ill., Urbana). It is postulated that conditions of environmental stress activate some biological control mechanism that inhibits nitrate reductase activity which in turn results in the accumulation of nitrate in the plants.

It is commonly accepted that nitrates are not toxic to plants while the intermediates from nitrite to ammonia are toxic within limits. Corn plants grown under high nitrogen fertility accumulate appreciable quantities of the amides, glutamine, and asparagine (10). According to Prianischnikov, amide formation (asparagine) is a mechanism for detoxification of ammonia (1). It is proposed that nitrate reductase and its biological control mechanism provide the plant with a second system for the regulation and control of toxic nitrogenous products. The question arises as to whether the differing abilities of various corn varieties to reduce nitrate under artificial or competitive plant shade (12) may be related to the relative proportions and effectiveness of these two protective systems, present in each hybrid.

The data presented in figures 1 to 5, permit the conclusion that the level of nitrate reductase activity (computed on basis of activity/g fresh wt, mg of protein, or shoot) was higher in the hybrid Hy2 × Oh7 than it was in the hybrid WF9 × C103. Assays made throughout the growing season on Hy2 × Oh7 plants grown under field conditions also support this conclusion. This higher level of nitrate reductase activity in the Hy2 × Oh7 strain was associated with a higher content of water soluble protein when the plants were grown under field, greenhouse, or constant temperature chamber conditions. Assays made on field-grown corn plants show that nitrate content correlates negatively with the nitrate reductase activity. Knipmeyer (12) showed that WF9 × C103 plants accumulated more nitrate when shaded than did the Hy2 × Oh7 plants. The consistent differences between these two strains in nitrate metabolism are attributed to the genetic characteristics of each strain.

Summary

Experiments were carried out to determine the effect of light and nitrate supply on the activity of nitrate reductase in two single cross corn hybrids (Hy2 × Oh7 and WF9 × C103). Plants were grown in the greenhouse or in a constant temperature room in soil, sand, or vermiculite cultures.

Young corn plants placed in complete darkness for 48 hours lost 90% of their nitrate reductase activity. The activity was quickly restored when the plants were returned to the light. In other experiments, corn plants were grown under artificial shade in the greenhouse. The nitrate reductase activity in these plants decreased roughly in proportion to the amount of shading. It was shown that both light and nitrate are necessary for the formation of nitrate reductase in quantities required by the plant for normal growth. Corn seedlings germinated in the dark in Hoagland solution showed a fourfold increase in nitrate reductase within 8 hours after they were transferred into the light. Plants that were germinated in the dark without nitrate, and given nitrate at the same time they were transferred to the light, showed a slower rate of development of nitrate reductase activity. Greenhouse experiments in which the nitrate content of nutrient media was varied showed that the level of nitrate reductase activity was dependent in part upon the substrate concentration.

There was a positive correlation between the nitrate reductase activity and the growth and protein content of both strains. This correlation supports the contention that the nitrate reductase activities measured are a reflection of the in vivo activities.

Shoots and leaves of the hybrid Hy2 × Oh7 have a higher level of nitrate reductase activity and protein content than similar tissue from WF9 × C103 plants.

Literature Cited

1. Bonner, J. 1950. Plant Biochemistry. 1st Edit. Pp. 1–537. Academic Press, New York.
2. Burstrom, H. 1943. Photosynthesis and assimilation of nitrate by wheat leaves. Ann. Rev. Agr. Coll. Sweden II. 1–50.
3. Candella, M. I., E. G. Fisher, and E. J. Hewitt. 1957. Molybdenum as a plant nutrient. X. Some factors affecting the activity of nitrate reductase in cauliflower plants grown with different nitrogen sources and molybdenum levels in sand culture. Plant Physiol. 32: 280–288.
4. Cheniae, G. and H. L. Evans. 1956. Nitrate reductase from the nodules of leguminous plants. In: Inorganic Nitrogen Metabolism, W. D. McElroy and B. Glass, eds. Pp. 184–188. John Hopkins Press, Baltimore.
5. Delwiche, C. C. 1951. The assimilation of ammonia and nitrate ions by tobacco plants. Jour. Biol. Chem. 189: 167–175.
6. Evans, H. J. and A. Nason. 1953. Pyridine nucleotide-nitrate reductase from extracts of higher plants. Plant Physiol. 28: 233–254.
7. Frear, D. S. and R. C. Burrell. 1958. The assimilation of N^{15} from labeled hyponitrite by soybean leaves. Plant Physiol. 33: 105–109.

8. HAGEMAN, R. H. and D. I. ARNON. 1955. Changes in glyceraldehyde phosphate dehydrogenase during the life cycle of a green plant. Arch. Biochem. Biophys. 57: 421–436.
9. HAGEMAN, R. H. and E. R. WAYGOOD. 1959. Methods for the extraction of enzymes from cereal leaves with especial reference to the triosephosphate dehydrogenases. Plant Physiol. 34: 396–400.
10. HAY, R. E., E. B. EARLEY, and E. E. DETURK. 1953. Concentration and translocation of nitrogen compounds in the corn plant (*Zea mays*) during grain development. Plant Physiol. 28: 606–621.
11. HOAGLAND, D. R. and D. I. ARNON. 1950. The water culture method for growing plants without soil. Univ. Cal. Agri. Expt. Sta. Cir. 347.
12. KNIPMEYER, J. W. 1958. The effect of light intensity on certain metabolites of *Zea mays*. M. S. Thesis, University of Illinois, Urbana.
13. MENDEL, J. L. and D. W. VASSAR. 1951. Studies on nitrate reduction in higher plants. Arch. Biochem. Biophys. 32: 158–169.
14. NASON, ALVIN. 1956. Enzymatic steps in the assimilation of nitrate and nitrite in fungi and green plants. In: Inorganic Nitrogen Metabolism. W. D. McElroy and B. Glass, eds. Pp. 109–136. John Hopkins Press, Baltimore.
15. NICHOLAS, D. J. D. and H. M. STEVENS. 1956. The role of molybdenum in oxidation-reduction processes in Neurospa and Azotabacter. In: Inorganic Nitrogen Metabolism, W. D. McElroy and B. Glass, eds. Pp. 178–183. John Hopkins Press, Baltimore.
16. SPENCER, D. 1959. A DPNH-specific nitrate reductase from germinating wheat. Australian Jour. Biol. Sci. 12: 181–196.
17. UMBREIT, W. W., R. H. BURRIS, and J. F. STAUFFER. 1957. Manometric Techniques. 3rd Edit. Pp. 1–338. Burgess, Minneapolis.
18. WOOLLEY, J. T., G. P. HICKS, and R. H. HAGEMAN. 1960. Rapid determination of nitrate and nitrite in plant material. Jour. Agr. & Food Chem. In press.

OXIDATION AND PHOSPHORYLATION BY MITOCHONDRIA FROM GREEN STEMS [1, 2, 3]

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Etiolated plants or very young seedlings generally are used as a source of mitochondrial preparations (5, 7). For example, Millerd et al (9), who apparently were first to isolate mitochondria capable of phosphorylation from plants, used etiolated mung bean seedlings. It is desirable for various reasons to start with green plants, but satisfactory preparations have been difficult to obtain (2). Recently, however, preparations oxidizing tricarboxylic acid cycle intermediates were prepared from green leaves (4, 6, 12, 14, 15) and phosphorylation was demonstrated (14, 15). In the present work, particulate fractions were obtained from green stems of tomato; the preparations were superior in several respects to those from etiolated tomato plants (11) and from green leaves (12). Ratios of phosphorus esterified to oxygen consumed were about 3.0. A hexokinase-glucose trapping system was required for phosphorylation. The preparation was relatively insensitive to 2, 4-dinitrophenol (DNP). Catalytic amounts of Krebs cycle acids were required for pyruvate oxidation. High activities of mitochondria from stems make these preparations useful for further experimentation, such as determination of biochemical changes associated with disease development (Wu, Lung-Chi and R. P. Scheffer. 1960. Comparative activities of mitochondria from Fusarium-infected and healthy tomato plants. In manuscript).

MATERIALS AND METHODS

Tomato plants of the variety, Bonny Best, were grown in the greenhouse in peat-sand mixtures and were fertilized with a suitable concentration of a complete nutrient solution. Greenhouse temperature was held at a minimum of 20° C. Plants were grown during the fall and winter for 4 to 6 weeks before harvesting, at which time they were 5 to 12 inches high. Stems were excised above the cotyledons and leaves were removed. The top 4 to 6 inches of long stems was used, unless indicated otherwise.

Pierpoint's procedure (12) was slightly modified for isolation of mitochondria. Stems were cut into pieces about one centimeter long and were chilled at 2° C for 10 minutes. The pieces were then placed in a mortar with half their weight of acid-washed sea sand and with a volume of extraction fluid twice the weight of the stems (ml–g). The material was ground vigorously for 2 minutes, then filtered through six layers of cheesecloth. The pH of the extraction medium did not change while grinding stems.

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[3] Michigan Agricultural Experiment Station Journal Article No. 2592.

The filtrate was centrifuged for 10 minutes at 1000 G and the pellet was discarded. The supernatant was re-centrifuged for 30 minutes at 10,000 G and the pellets containing the mitochondria were suspended in washing medium equal in volume to the original weight of the stems (ml–g). After a second centrifugation at 10,000 G for 30 minutes, the supernatant was discarded and the final washed pellet was suspended in an adequate volume of 0.25 M sucrose solution for the enzymic studies. All preparation was done in a cold room at about 2° C, using previously chilled glassware and solutions. In a typical experiment, 30 g of stems were ground with 15 g sand and 60 ml extraction medium. After high speed centrifugation, the pellet was washed with 30 ml washing medium, and after final centrifugation was suspended in 1.5 ml sucrose solution. When larger quantities were required, stems were ground in batches of 30 g each.

Pierpoint's extraction and washing media (12) were used. The extraction solution contained the following: sucrose, 0.4 M; tris (hydroxymethyl) aminomethane (tris buffer), 0.2 M; ethylenediamine tetraacetic acid (EDTA), 0.005 M; dibasic potassium phosphate, 0.01 M; and sodium citrate, 0.02 M. The solution was adjusted to pH 7.9 with 5 *N* hydrochloric acid. Washing medium contained sucrose, 0.2 M; tris buffer, 0.1 M; EDTA, 0.0025 M; dibasic potassium phosphate, 0.005 M; and sodium citrate, 0.005 M. Final pH was 7.3.

The Warburg apparatus was used to determine oxygen uptake, following standard procedures (16). Flasks containing the reaction medium were chilled before the mitochondrial suspension was added. Equilibration time was 10 minutes, except when P : O ratios were determined, when the time was shortened to 5 minutes. All experiments were run at 30° C. Each flask contained 2.0 ml total volume of reaction mixture, including 0.4 ml mitochondrial suspension. The pH of reaction mixture in all cases was 7.3. The following materials and final concentrations in flasks were used as required in the various experiments: substrates, (potassium salts) 0.025 M; sucrose, 0.2 M; monobasic potassium phosphate, 0.07 M; magnesium sulfate, 0.0075 M; adenosine triphosphate (ATP), 0.001 M; cytochrome c, 0.1 mM; diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide, (TPN) 0.13 mM; thiamine pyrophosphate (TPP), 0.13 mM; coenzyme A (CoA), 0.013 mM; malonate, 0.025 M. For P : O ratio determinations, 0.2 ml hexokinase solution (containing 2 mg/ml type IV in 0.5 M glucose) was added to the flask side arm and tipped into the reaction mixture after equilibration. All enzymes, co-factors, and nucleotides were obtained from Sigma Chemical Co.

Inorganic phosphate (Pi) was determined by the method of Fiske and Subbarow (3). Two ml of chilled, 10 % trichloroacetic acid (TCA) was added to the reaction fluid in each Warburg flask, and a 2 ml sample of the resulting mixture was removed for determination of Pi. Samples were centrifuged at 1500 G for 15 minutes at 2° C to remove precipitates, and 1 ml of the supernatant was diluted with 9 ml of chilled water. From this dilution, 0.2 ml samples were removed and added to 8.4 ml water, to which was further added 1 ml 2.5 % ammonium molybdate and 0.4 ml 0.25 % aminonaphtholsulfonic acid. After a 15 minute incubation time, color development was measured at 660 mμ (No. 66 filter) with a Klett-Summerson colorimeter. Two Pi samples were run for each Warburg flask. Separate flasks were used to determine Pi at the beginning of each experiment; after equilibration in the Warburg water bath, TCA was added to the mixture before hexokinase was tipped in. This order of procedure was necessary for maximum precision.

Protein nitrogen in mitochondrial preparations was determined by Ma and Zuazaga's (8) micro-Kjeldahl method, slightly modified. One milliliter samples of mitchondrial suspensions were dried at 100° C and washed by centrifugation first with 10 ml of 15 % (W/V) TCA, then with 10 ml 7.5 % TCA. Centrifugation was at 1,500 G for 15 minutes at room temperature. The pellet was dissolved in 1 ml of 1 *N* sodium hydroxide, to which was added 1 ml water. Samples (0.4 ml) of this solution were digested with 3 mg selenium, 5 mg potassium sulfate-copper sulfate mixture, and 1 ml concentrated sulfuric acid for 20 minutes. The reaction mixture was distilled into 2 % boric acid and titrated with 0.01 *N* hydrochloric acid to measure ammonia released.

Results

Succinoxidase Activity: The proper isolation procedure was determined in preliminary experiments by using succinate as the substrate for testing activity. Chilling stems before grinding was found to be desirable, but variation in grinding time from 2 to 5 minutes had little effect. The volume of extraction medium affected activity, as Pierpoint (12) found for preparations from tobacco leaves. The ratio of extraction medium to sea sand to washing medium to

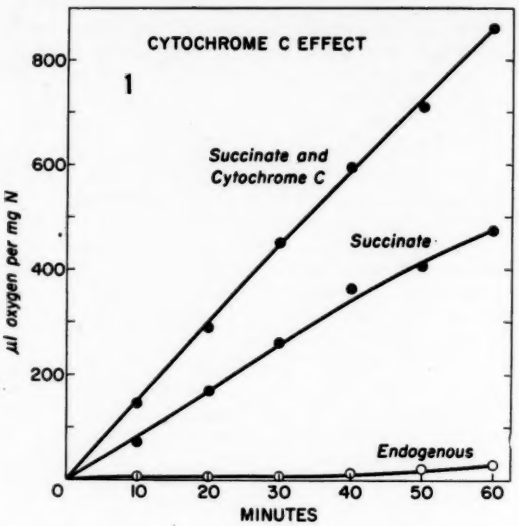
Table I

Succinoxidase Activity of Mitochondria from Tomato Stems

Experiment*	Oxygen uptake**			
	Endogenous		Succinoxidase	
	30 min	60 min	30 min	60 min
1	13	33	150	212
2	46	70	295	521
3	19	30	312	552
4	12	19	288	495
5	0	12	224	425
6	10	15	210	420

* Reaction mixture (pH 7.3) contained the following, in μ moles per flask: potassium succinate, 50; KH_2PO_4, 140; sucrose, 400; $MgSO_4$, 15; ATP, 2.

** Oxygen uptake in μl per mg protein nitrogen.



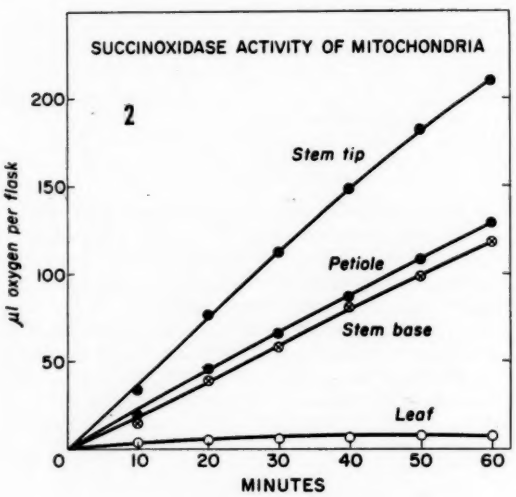


FIG. 1. Effect of cytochrome c on succinoxidase activity of mitochondria from tomato stems.

Reaction mixture contained the following, in μ moles per flask: sucrose, 400; KH_2PO_4, 140; $MgSO_4$, 15; and ATP, 2. 50 μ moles potassium succinate and 0.2 μ moles cytochrome c were used in flasks indicated.

FIG. 2. Succinoxidase activity of mitochondrial preparations from stems, petioles, and leaves. Endogenous oxygen uptake, which was high for the leaf preparation, was subtracted to obtain the values given. The reaction mixture (pH 7.3) contained the following, in μ moles per flask: sucrose, 400, potassium succinate, 50; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; and cytochrome c, 0.2.

fresh weight of stems finally used was 2:0.5:1:1. One extra 30 minute centrifugation of mitochondria in the extraction medium caused a 50 % decrease in succinoxidase activity.

After proper isolation procedure was found, a preparation showing high succinoxidase activity was obtained (table I). Activity and yield were fairly consistent from experiment to experiment, even though tomato plants of different ages and stem textures were used. Succinoxidase activity was further increased by 45 to 80 % by adding 0.2 μ moles cytochrome c in each flask (fig 1). Response to cytochrome c seemed to vary with the age of the plants used as source material. Maximum $Qo_2(N)$ in a number of experiments was 873 μl. This high oxygen uptake was obtained without the addition of co-factors to the reaction medium. In many of the experiments described below, both cytochrome c and co-factors were added. Mitochondrial yields were 0.510 to 0.645 mg protein N per 20 g green stems.

The effect of 2, 4-dinitrophenol (DNP) on succinoxidase activity by the mitochondria was determined by using DNP in concentrations from 5×10^{-5} M to 10^{-6} M. Treatment with 10^{-6} M (0.002 μ moles/flask) DNP caused a 15 % increase in oxygen uptake over the untreated control and 5×10^{-6} M caused a 12 % increase, when used without cytochrome c. Concentrations greater than 5×10^{-6} M inhibited oxygen uptake in all cases.

The effect of malonate on succinoxidase activity was tested, using a reaction mixture which was otherwise the same as that given in table I. Succinoxidase activity was inhibited 88 to 90 % by 0.025 M (50 μ moles/flask) malonate.

Experiments were designed to determine the best part of the stem to use as source material, and to compare the succinoxidase activities of stem and green leaf preparations. Plants 12 inches high were separated into several fractions of equal fresh weight; the top 2 inches of the stem was used as one part, a 2.5 inch piece in the second internode above the cotyledons was used as another part, the petioles of the top expanded leaves were used as a fourth part. Mitochondrial preparations were made in the usual way. The young stem tips gave the best preparation, containing more protein N and giving higher oxygen uptake than the other fractions (fig 2). Preparations from the lower stem contained less protein N in the final mitochondrial suspension, and had correspondingly less oxygen uptake. Preparations from both lower and upper petioles were about equal to those from stem bases. The leaf preparation was not considered satisfactory (fig 2).

The preparation from stems had a pale green color, indicating a chlorophyll contamination. Chlorophyll in stems and in the preparation was estimated as described by Pierpoint (12). Total chlorophyll in 60 g of stems was about 3.2 mg, and the mitochondrial preparation had less than 10 % of the starting chlorophyll. These levels are far less than those found in leaves and in preparations from leaves (12).

TABLE II

OXIDATION OF KREBS CYCLE INTERMEDIATES BY MITOCHONDRIA FROM TOMATO STEMS

SUBSTRATE*	% OF SUCCINOXIDASE ACTIVITY**	
	WITHOUT CO-FACTORS	WITH CO-FACTORS***
Succinate	100	...
Citrate	15.8	31.1
α-Ketoglutarate	14.4	37.9
Fumarate	2.7	11.0
Malate	21.2	73.2

* Reaction mixture (pH 7.3) contained the following in μ moles per flask: substrates (K salts), 50; sucrose, 400; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; and co-factors as required.

** Determined at 30 minutes reaction time.

*** Co-factors: 0.26 μ moles each of DPN and TPP, and 0.026 μ moles CoA. Succinate without co-factors was used as the base for all comparisons.

OXIDATION OF OTHER INTERMEDIATES: Potassium salts of malate, fumarate, α-ketoglutarate, and citrate were used as substrates in two sets of experiments. These substrates were used with and without co-factors (DPN, TPP, and CoA). Results were similar to those reported by Pierpoint (12) for mitochondria from tobacco leaves, except that the stem preparation had a higher activity per unit nitrogen and a lower endogenous respiration. In all cases the addition of co-factors increased oxygen uptake (table II). Malate was the most readily oxidizable of these substrates, followed by α-ketoglutarate and citrate; fumarate was utilized less readily than were the others (table II).

DPN and TPN as co-factors were compared, using citrate and malate as substrates. DPN alone gave a striking increase in oxygen uptake (table III), reaching a level which was not further increased by adding TPN as a second factor. TPN alone supported a small but significant increase in oxygen uptake, as compared to a reaction mixture containing neither TPN nor DPN. DPN was added to the medium in later experiments.

PYRUVATE OXIDATION: An important criterion for mitochondrial preparations is the failure to oxidize pyruvate unless catalytic amounts of Krebs cycle acids are present. The mitochondria from the stems were examined for this effect by using a pyruvate-malate system. Pyruvate alone was a very poor substrate, but it was oxidized readily when a small amount (2.5 μ moles/flask) of malate was added (fig 3). Pyruvate plus malate gave oxygen uptake about double the expected additive effect of malate and pyruvate used singly. These results confirm previous work (9, 12). However, the green tomato stem

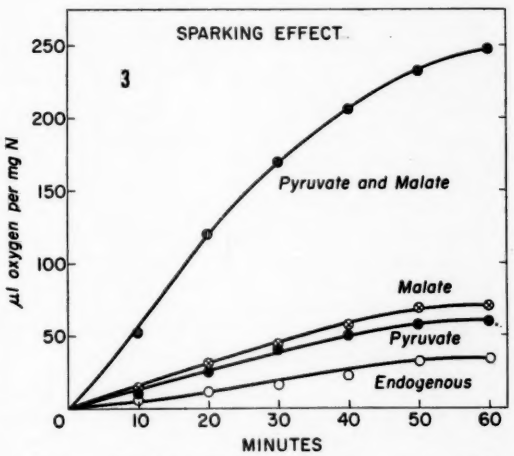


FIG. 3. Catalytic effect of malate on pyruvate oxidation by mitochondria from tomato stems.

Reaction mixture contained the following, in μ moles per flask: sucrose, 400; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; TPP, 0.26; DPN, 0.26; CoA, 0.026; pyruvate 50 and malate, 2.5.

TABLE III

EFFECT OF DPN AND TPN ON OXIDATION OF CITRATE AND MALATE BY MITOCHONDRIA FROM TOMATO STEMS

SYSTEM	CITRATE		MALATE	
	O_2 UPTAKE*	% INCREASE OVER CONTROL	O_2 UPTAKE*	% INCREASE OVER CONTROL
Control**	25.4	...	32.5	...
+ TPN***	35.7	41	41.1	26
+ DPN***	77.2	204	125.2	285
+ TPN & DPN	76.5	201	128.2	294

* O_2 uptake in μl per flask per 60 minutes.

** Control reaction mixtures contained the following, in μ moles per flask; sucrose, 400; substrates (K salts), 50; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; TPP, 0.26; and CoA, 0.026. pH was 7.3.

*** DPN and TPN were used at 0.26 μ moles per flask.

TABLE IV

INORGANIC PHOSPHORUS AND OXYGEN UPTAKE BY MITOCHONDRIA FROM TOMATO STEMS

EXPERIMENT*	PI UPTAKE**	OXYGEN UPTAKE***	P:O RATIO
1	11.0	4.74	2.32
2	17.3	6.21	2.79
3	23.5	8.25	2.85
4	26.3	10.8	2.44
5	25.0	7.8	3.2
6	25.2	8.82	2.86
7	20.0	6.82	2.93

* Reaction mixture contained the following in μ moles per flask: α-ketoglutarate, 50; sucrose, 400; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; DPN, 0.26; TPP, 0.26; CoA, 0.026; malonate, 50; and 100 μ moles glucose containing 2 mg per ml type IV hexokinase. pH was 7.3.

** Pi uptake in μ moles per flask. Values are averages of six determinations.

*** Oxygen uptake in μ atoms per flask. Values are averages of three flasks each. Reaction times varied with the experiment from 30 to 50 minutes.

preparation differed from the preparation from green tobacco leaves (12) in that the leaf preparation did not oxidize pyruvate, even in the presence of Krebs cycle acids, without pre-treatment with dilute ammonia. A pH effect during grinding was ruled out as an explanation of the difference between the tomato and tobacco preparations.

OXIDATIVE PHOSPHORYLATION: The significant criterion of mitochondrial preparations is the incorporation of inorganic phosphate (Pi) into energy rich phosphate compounds. This effect was tested for the preparation from green tomato stems by using α-ketoglutarate as a substrate and inhibiting the cyclic reactions beyond succinate by adding malonate to the reaction mixture. Hexokinase was used to complete the system. Results showed that Pi was taken up, with P:O ratios between 2.3 and 3.2 (table IV). Very likely ATP was formed in this process, as Millerd et al (9) have shown, but ATP and glucose-6-phosphate isolations were not attempted in these experiments.

Further experiments showed that 5×10^{-6} M DNP (0.01 μ moles/Warburg flask) depressed Pi uptake slightly (table V) when used at pH 7.3. Much more striking, however, was the hexokinase effect. When this enzyme was omitted from the reaction mixture, Pi uptake and oxygen uptake were decreased, and the P:O ratio was 16 to 30 % of the value obtained with hexokinase. The experiment summarized in table V was repeated three times with essentially the same results. Thus oxidation by the mitochondrial preparation from tomato stems is coupled very well with phosphorylation.

DISCUSSION

Mitochondrial preparations from tomato stems contained a small amount of chlorophyll, but no visible chloroplasts. Nevertheless, the possibility of chlorophyll affecting the reactions was considered. In the past some workers believed that chloroplasts contained oxidative enzymes generally found in mitochondria (10). More recent data show that chloroplasts are not able to oxidize Krebs cycle intermediates (1, 6), while mitochondria from green leaves oxidized the intermediates and catalyzed respiratory chain phosphorylation in which no light induced reaction was involved (6, 14, 15). Thus, the traces of chlorophyll in our preparations probably were of no significance.

Succinoxidase activity of the particles from green tomato stems was much higher than the activity reported for preparations from etiolated tomato hypocotyls (11). After addition of cytochrome c the preparation was still more active, comparing favorably with Smilliés (14, 15) which is the most active reported for plants. A cytochrome c dependent increase in activity was reported by Pierpoint (12) for tobacco leaf preparations, but particles from etiolated tomato hypocotyls did not respond to cytochrome c (11). The cytochrome c response may be related to mitochondrial damage, as suggested by others (9).

Other substrates and inhibitors were used to determine whether or not the stem preparation behaved as expected for mitochondria. Malate, fumarate, α-ketoglutarate, and citrate were oxidized more slowly than was succinate. Addition of co-factors (DPN, TPP, and CoA) increased the oxidation rate in all

TABLE V

EFFECT OF 2, 4-DINITROPHENOL AND HEXOKINASE ON PHOSPHORYLATION BY MITOCHONDRIA FROM TOMATO STEMS

SYSTEM	PI UPTAKE*	OXYGEN UPTAKE**	P:O RATIO	% OF CONTROL RATIO
Complete***	26.0	10.8	2.41	100
$+5 \times 10^{-6}$ M DNP	23.8	10.97	2.17	90.0
Less Hexokinase	2.3	5.97	0.38	16.0

* Pi uptake in μ moles per flask. Values are average of six determinations.

** Oxygen uptake in μ atoms per flask. Values are averages of three flasks each.

*** Complete reaction mixture contained the following in μ moles per flask: sucrose, 400; α-ketoglutarate, 50; KH_2PO_4, 140; $MgSO_4$, 15; ATP, 2; cytochrome c, 0.2; DPN, 0.26; TPP, 0.26; CoA, 0.026; malonate, 50; and glucose, 100. Hexokinase, (Sigma Type IV, 0.2 ml containing 2 mg/ml) was added with the glucose. pH was 7.3.

cases. Pyruvate was a very poor substrate when used alone, but was oxidized readily when a catalytic amount of malate was provided. Succinoxidase activity was inhibited 80 % to 90 % by malonate used in the usual concentration for such work. The stem preparation apparently reacts the same in these respects as mitochondria from other sources.

Oxidation of ketoglutarate in the presence of hexokinase and glucose gave active phosphorus uptake, with P : O ratios from 2.3 to 3.2. Such values indicate good coupling between phosphorylation and oxidation. When only hexokinase was omitted from the reaction mixture, Pi uptake, oxygen uptake, and P : O ratios were reduced drastically. Since mitochondria from plants generally are thought to contain adequate hexokinase, the tomato stem preparation seems to be unusual in this respect. The preparation is unusual also in the relative insensitivity to DNP, a situation previously described only for particles from avocado (13). The hexokinase requirement indicates that ATP is formed during oxidation of ketoglutarate, since hexokinase requires ATP to phosphorylate glucose; no other mechanism is known for this reaction. Thus ADP is regenerated from ATP by the transfer of P to glucose by hexokinase, and phosphorylation can continue. Phosphorus acceptors limit respiration in many biological systems (7).

The fact that very satisfactory mitochondria were obtained from green stems deserves emphasis, because only Smillie (14) has reported on the use of stems. Preparations were made from young and old parts of the stem, and activities were compared with preparations from leaves and petioles. Of all sources, young stem tips gave the best preparation, containing more protein nitrogen with slightly higher activity per unit nitrogen. Leaf blade preparations were sticky and contained high levels of chlorophyll and nitrogen, as Pierpoint (12) found for tobacco leaves. Tomato leaves were used several times without obtaining a satisfactory preparation; endogenous respiration was high and there was little increase after substrate was added. A better preparation may be possible, but leaves were not used in further work.

Summary

Particles isolated from green stems of tomato possessed the essential properties of mitochondria. Malate, succinate, fumarate, citrate, and α-ketoglutarate were oxidized, and the co-factors DPN, TPP, and CoA increased the rates of oxidation. Pyruvate served as a very poor substrate unless a catalytic amount of a Krebs cycle acid was added to the reaction mixture. Succinoxidase activity was among the highest known for plant mitochondria. Oxidation was coupled with phosphorylation, and P : O ratios between 2.3 and 3.2 were obtained with ketoglutarate. Phosphorylation depended on the addition of hexokinase to the reaction mixture. The preparation was relatively insensitive to DNP.

Acknowledgment

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Literature Cited

1. Arnon, D. I., M. B. Allen, and F. R. Whatley. 1956. Photosynthesis by isolated chloroplasts. IV. General concept and comparison of three photochemical reactons. Biochem. Biophys. Acta 20: 449–461.
2. Brummond, D. O., and R. H. Burris. 1954. Reactions of the tricarboxylic acid cycle in green leaves. Jour. Biol. Chem. 209: 755–765.
3. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorus. Jour. Biol. Chem. 66: 375–400.
4. Freebairn, H. T., and L. F. Memmert. 1956. Oxidative activity of subcell particles from a number of plant species. Plant Physiol. 31: 259–266.
5. Hackett, D. P. 1955. Recent studies on plant mitochondria. Intern. Rev. Cytol. 4: 143–196.
6. James, W. O., and V. S. R. Das. 1957. The organization of respiration in chlorophyllous cells. New Phytol. 56: 325–343.
7. Laties, G. G. 1957. Respiration and cellular work and the regulation of the respiration rate in plants. Survey of Biol. Progress 3: 215–299.
8. Ma, T. S., and G. Zuazaga. 1942. Micro-Kjeldahl determination of nitrogen. Ind. Eng. Chem. 14: 280–282.
9. Millerd, Adele, J. Bonner, B. Axelrod, and R. Bandurski. 1951. Oxidative and phosphorylative activity of plant mitochondria. Proc. Nat. Acad. Sci. U. S. 37: 855–862.
10. Ohmura, T. 1955. Oxidative phosphorylation by a particulate fraction from green leaves. Arch. Biochem. Biophys. 57: 187–194.
11. Paquin, R., and E. R. Waygood. 1957. The effect of fusarium toxins on the enzymic activity of tomato hypocotyl mitochondria. Can. Jour. Bot. 35:207–218.
12. Pierpoint, W. S. 1959. Mitochondrial preparations from the leaves of tobacco (*Nicotiana tabacum*). Biochem. Jour. 71: 518–528.
13. Romani, R. J. and J. B. Biale. 1957. Metabolic processes in cytoplasmic particles of the avocado fruit. IV. Ripening and the supernatant fraction. Plant Physiol. 32: 662–668.
14. Smillie, R. M. 1955. Enzymic activity of particles isolated from various tissues of the pea plant. Australian Jour. Biol. Sci. 8: 186–195.
15. Smillie, R. M. 1956. Enzymic activities of subcellular particles from leaves. I. The occurrence of mitochondria in green leaves of the pea plant. Australian, Jour Biol. Sci. 9: 81–91.
16. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques, Ed. 3. 338 pp. Burgess Publ. Co., Minneapolis.

METABOLISM OF ISOLATED LEAVES: I. CHANGES IN THE PROTEIN, SOLUBLE NITROGENOUS COMPOUNDS, SUGARS, AND ORGANIC ACIDS IN TOBACCO LEAVES IN LIGHT AND DARK[1, 2]

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Degradation of protein level in detached leaves has long been known and the accumulation of free amino acids and amide at the expense of protein breakdown has been evident from the time of Schulze (1898) followed by Chibnall (6), Mothes (cf. 6), Yemm (42), Vickery et al (33), Mc Kee (18), Wood et al (41), and Viets et al (38). However the changes in individual amino acids remained uninvestigated because of the lack of proper methods. During recent years paper chromatography has been widely applied as a tool for biochemical investigations and recently an attempt was made to study the amino acid metabolism of leaves after removal from the plant under conditions of light and dark (23, 24).

The difficulty in detecting some of the overlapping amino acids by the horizontal migration method necessitated examination employing two-dimensional chromatography of amino acids.

Since protein metabolism is closely related to other metabolic activities (6, 12) and hence is difficult to interpret without knowledge of the other processes, it was of interest to follow the changes in the other two important fractions, the sugars and the organic acids, simultaneously occurring in detached leaves. In this way it was hoped to obtain a clear picture of metabolic events operative during the culture of detached leaves. *Nicotiana tabacum* L. was selected as the working material, for its metabolic behavior is known through the work of Vickery and his colleagues (33), and it has been found to exhibit characteristic metabolism not only in relation to protein, amino acids, and amides but also with respect to organic acids.

Although the loss of malic acid and gain of citric acid has repeatedly been confirmed for Connecticut tobacco leaves cultured in the dark (33, 34, 35, 36), it becomes, however, difficult to explain these results if the normal citric acid cycle (16) is considered operative in these leaves. At the most it could be explained by assuming that in detached leaves cultured in the dark the rate of synthesis of citric acid from malic acid exceeded that of the decomposition of citric acid. How darkening of detached leaves would foster these changes is a matter of speculation. Organic acids of the tricarboxylic acid cycle are not normally consumed for meeting the energy requirements of the tissue, they being intermediate oxidation products. Chibnall (6), while calling it an "organic acid phase" in the metabolism of starving tobacco leaves, strongly felt that such a behavior would be most unlikely if the normal Krebs cycle is considered operative. In the latter case the levels of the respective acids should have remained unchanged. Regarding the possibility of an organic acid cycle participating in Nicotiana leaves to meet its energy requirements, Chibnall (6) himself wrote: "The interpretation that I have given to these five sets of starvation experiments is admittedly speculative in that it is based on the hypothesis of an organic acid cycle in respiration for which there is no ad hoc proof at present and perhaps there never can be" (p. 241).

This conclusion was, of course, due to lack of the knowledge of pathways of malic-citric conversion involving enzyme systems other than those in the Krebs cycle at that time.

Experiments have been conducted to elaborate the pathway of malic-citric conversion in Nicotiana leaves. The success has been mainly due to the finding that the leaves cultured in streptomycin and penicillin solution in dark failed to show the conversion, which *Nicotiana tabacum* var. Cheroot leaves carried out when cultured in water in the dark. This observation made it necessary to examine whether the organic acid metabolism of variety S-20 leaves which did not show this conversion, could also be altered by culturing on antibiotic solution, and whether the enzymes for malic-citric conversion of the regular citric acid cycle were operative in these leaves. The latter has been demonstrated by the simple method of feeding the leaves with organic acid salts (succinate and citrate).

MATERIALS AND METHODS

MATERIAL: Two varieties of *Nicotiana tabacum*, S-20 and Cheroot, have been used. The plants were raised from seeds obtained from the Central Tobacco Research Station, Rajahmundry, Andhra. The seedlings were raised in earthenware pots rich in manure and at the four leaf stage they were transplanted to the experimental plots of the Agricultural Farm of the Botany Department, University of Allahabad, in one season and in the plots of the Botanical Gardens in the other season. The experiments were conducted with full-grown plants 1½ months old.

[1] Received revised manuscript March 9, 1960.

[2] Part of the thesis approved for the Doctor of Philosophy degree of the University of Allahabad (1957) and supported by the Empress Victoria Readership of the University of Allahabad and by the Government of India Senior Research Training Scholarship to Manmohan M. Laloraya.

About 50 leaves were collected for each experiment from tobacco plants, grown to a uniform size, from the fourth node down from the apex. These were then sampled on the basis of the leaf size, usually falling into three groups. However, the largest number of leaves in such a group were of average size and these were selected for the experiment; the bigger and the smaller leaves were discarded. The leaves were washed in running tap water, blotted gently, and exposed to a fan for 15 to 20 minutes to remove all the surface water, and weighed.

Three leaves from such a group were analyzed separately for the free amino acids, sugars, and organic acids to check the uniformity of such a sample. Chromatographic analyses gave a uniform picture within this triplicate sample. It was thus assumed that leaves falling into this group were not only uniform in regard to growth but also that they were closely identical in their metabolic state.

The desired number of leaves were placed with their petioles in distilled water, which was changed every 2nd day, in a dark chamber at room temperature (22° C ± 2° C) and a similar other set was exposed to diffused day light during the day and to a 9,000 lux during the night from two 500 w Osram lamps. A water jacket was introduced between the lamps and the leaf chamber to absorb the excess heat. In the other experiment, from a set of 13 leaves, which were initially incubated in a dark chamber, four leaves were exposed to light after 174 hours of culture in the dark while the other set of four leaves was continued in the dark. The light treatment was diffuse daylight during the day and 6,000 lux at night from fluorescent daylight tubes.

An experiment with antibiotic solution was carried out under similar conditions. The antibiotic solution consisted of 0.1 % w/v streptomycin (Pfizer) and 400 lakhs units of penicillin (Lilly) dissolved in a liter of sterilized distilled water, mixed together in the ratio of 1 : 1. As the antibiotic solution was susceptible to fungal attack during continuous culture of leaves it was found desirable to change the antibiotic solution as well as water after every 48 hours during the experimental period.

For feeding experiments with the S-20 variety of tobacco leaves two active metabolites of the citric acid cycle were chosen. Succinate was given as sodium salt in 0.05 M concentration whereas potassium salt was used for citrate in equivalent concentration.

Methods

Extraction of Soluble Fractions: The extraction procedure was that described by Steward, Wetmore, Thompson, and Nitsch (30).

The leaves at different experimental periods were chopped quickly by a sharp razor and the cut pieces dropped in 80 % boiling ethanol. After boiling the leaf tissue for 10 to 15 minutes in alcohol, the alcohol was decanted and the leaf tissue ground with a mortar and pestle. The ground tissue that was transferred to the original alcoholic solution by further alcohol washings, was stirred for about an hour on an electromagnetic stirrer and then left overnight at room temperature. It was then filtered and the residue re-extracted, the filtrates being combined.

The 80 % alcoholic extract (containing amino acids, amides, free sugars, and organic acids) was evaporated on a hot water bath and the residue redissolved in a known volume of 20 % ethanol to make the final concentration of 1 ml extract per gram fresh weight of the leaf tissue. Soluble proteins which precipitated were removed by centrifugation. The clear liquid was decanted for chromatographic analysis while the colloidal proteins were added to the original residue kept for protein analysis.

Protein Fraction: The alcohol extracted residue, containing the protein nitrogen and the added colloidal protein, was hydrolyzed using 6 *N* HCl in the autoclave at 15 lbs pressure for 30 minutes. A pinch of stannous chloride was added to avoid humin formation and destruction of amino acids in the presence of carbohydrates. The hydrolyzed residue was filtered and the hydrolyzate so obtained was adjusted to volume making 1 ml of hydrolyzate per gram fresh weight of the leaf. Prior neutralization and desalting of the hydrolyzate for chromatographic assay was considered unnecessary, since introducing 0.5 % w/v ammonia in water-saturated phenol gave satisfactory chromatographic separations without any tailing of the spots. However, desalting of solutions was also avoided because certain amino acids, e.g., arginine, are reported to be destroyed during the electrolytic dissociation.

Chromatographic Methods; Amino Acid and Amide: The two-dimensional ascending chromatographic technique as described by Consdon, Gordon, and Martin (7) was followed.

Twenty-five microliters and ten microliters of the alcoholic extract and of protein hydrolyzate, respectively, were spotted 3 cm from the two edges of the 28 × 28 cm Whatman No. 1 filter paper for analysis of the free amino acids, amides, and the protein-bound amino acids, using Shandon micropipettes.

Partridge's (1948) solvent as modified by Fowden (10), namely phenol saturated with 0.5 % w/v ammonia solution, was used as the first running solvent; it took about 14 hours to traverse the length of the filter paper. The chromatograms, after drying for 2 days at room temperature (22° ± 2° C) to remove excess of phenol, were developed in the other direction with *n*-butanol : acetic acid : water (4 : 1 : 5) mixture. The time required for the second run was between 12 and 14 hours.

Ninhydrin at a concentration of 0.1 % (w/v) in *n*-butanol, was sprayed to locate the amino acid and amide spots. When dry the chromatograms were heated in an oven at 85° C for 30 minutes to develop the spots and were later exposed to room conditions for 30 minutes.

Proline, which gives a weak reaction with ninhydrin as a yellow spot, was confirmed by spraying with

the Isatin reagent of Saifer and Orekes (1954), drying at room temperature and heating at 100° C for 10 minutes. Proline gave an intense navy-blue spot.

Analysis of tryptophan was not made separately since it is destroyed during acid hydrolysis of protein.

QUANTITATIVE EVALUATIONS OF SEPARATED AMINO ACID AND AMIDES: The photometric method of Bull, Hahn, and Baptist (5) and of Block (3) based on the measurement of spot intensities, following treatment with ninhydrin, has been used.

However, since absolutely controlled conditions were lacking a strictly quantitative evaluation was impossible.

To avoid this discrepancy all the chromatograms in one set of experiments were developed at the same time under similar conditions. The spots were developed simultaneously and their density measured—each one common spot from all the chromatograms scanned at one time. In this way it was hoped to give as closely as possible the comparative values of different amino acids and sugars in terms of density changes. Visual observation of the spots were in conformity with the density measurements. Comparative quantitative values have been presented as the maximum density of amino acid spots produced following ninhydrin treatment for all amino acids using a red filter and recording by a Photovolt Densitometer at sensitivity three. For proline the same procedure was applied to the spot produced by the isatin reagent.

The paper blank was adjusted by calibrating the instrument to zero density or maximum light transmission using a piece of paper cut from the chromatogram. The cut areas of the respective amino acid spots were introduced below the photocell in such a position as to allow the pinhead light beam to pass through the center of the spot. The density values were recorded and the maximum density of each amino acid determined.

SUGARS: The horizontal migration multiple sector chromatographic technique of Ranjan et al (22) was followed using *n*-butanol : acetic acid : water (4 : 1 : 5) as solvent. Circular Whatman No. 1 special chromatographic filter paper discs of 28 cm diameter, divided into 12 equal sectors by radial fins clipped off at equal distances around the circumference of a circle of 3 cm diameter, traversing radially 12 cm of the filter paper disc, was used.

Ten μl of the alcohol extracts were spotted at their respective sectors. Double development of the chromatograms (The 1st for 6 hrs and the 2nd for 4 hrs) was carried out to achieve better separation of the sugars. The aniline-diphenylamine phosphate reagent of Buchan and Savage (4) was sprayed and the spots were developed by heating the dry chromatograms for 10 minutes at 80° C. The reagent offered the advantage that a direct photometric determination of the various sugars was made possible. The sectors were removed, before the background of the chromatogram turned black, and the density of the spots was measured at maximum sensitivity, three, using a yellow green filter in a Photovolt Densitometer.

ORGANIC ACIDS: The circular paper chromatographic technique of Ranjan and Rajarao (25) was followed. Two dimensional chromatography, employing ethanol : ammonia : water (95 : 5 : 5) as the first solvent and *n*-butanol : formic acid : water (10 : 2 : 5) as the second solvent, was also carried out to confirm the organic acids separated by the horizontal migration method. Bromophenol blue, 0.04 % w/v in ethanol was sprayed to detect the organic acids. Two and one half microliters of the alcoholic extract were spotted for analysis at each sector.

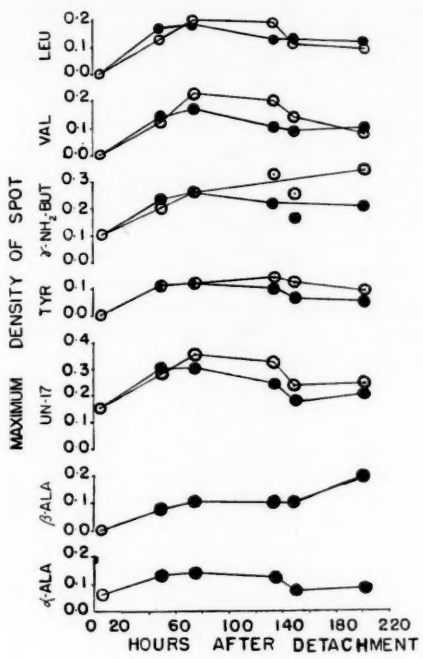
RESULTS

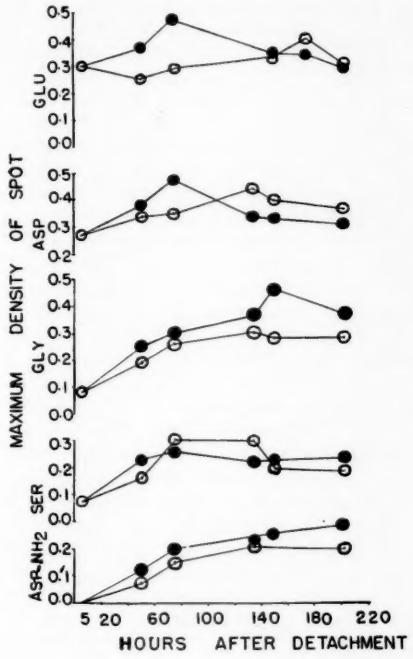
CHANGES IN S-20 VARIETY OF TOBACCO LEAVES IN LIGHT AND IN DARK: *Amino acids and amides.* Analysis of the protein of the tobacco leaf revealed the presence of leucine, isoleucine, phenylalanine, valine, tyrosine, proline, α-alanine, glutamic acid, threonine, aspartic acid, glycine, serine, arginine, histidine, and lysine. During culture in water both in light and dark, protein degradation was indicated by a general decrease of all the protein amino acids.

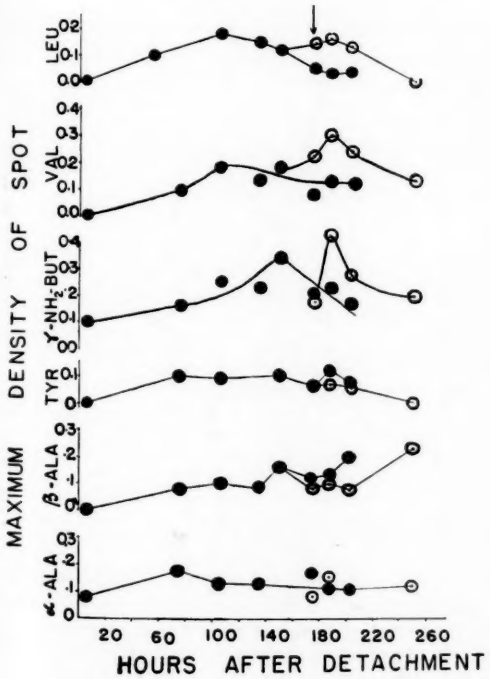
Amino acid analysis of the alcohol-soluble fraction 5 hours after the detachment of the leaf showed the presence of γ-aminobutyric acid, α-alanine, proline, glutamic acid, aspartic acid, serine, glycine, and cysteic acid. After 50 hours culture on distilled water in light and in dark a wide range of free amino acids made their appearance. The chromatogram showed the presence of leucine, isoleucine, phenyl-alanine, valine, γ-aminobutyric acid, tyrosine, proline, β-alanine, α-alanine, glutamic acid, threonine, arginine, aspartic acid, serine, glycine, asparagine, histidine, cysteic acid, and an unidentified ninhydrin positive substance. Subsequent analysis at different hours did not present any qualitative change in the free amino acids; the acids exhibited, however, marked changes, among themselves during the culture period.

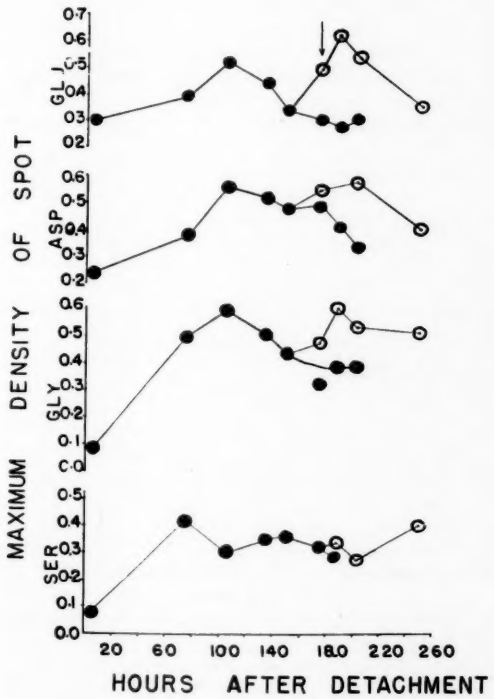
FIG. 1 (*top left*) and FIG. 2 (*top right*). Showing changes in the free amino acids and amide in isolated tobacco leaves cultured in water in the light and in the dark. Open circles, light; closed circles, dark.

FIG. 3 (*below left*) and FIG. 4 (*below right*). Showing the changes in the free amino acids in tobacco leaves exposed to light after an initial 174 hours of dark culture in water. Arrow indicates the time at which the leaves were transferred to light. Curves for asparagine and unidentified ninhydrin reacting substance 17 have not been shown. Exposure to light had no appreciable effect on these substances. Leu, leucines + phenylalanine; Val, valine; γ-NH_2-but.-γ-aminobutyric acid; Tyr, tyrosine; UN-17, unidentified ninhydrin reacting substance; β-ala, β-alanine; α-ala, α-alanine; Glu, glutamic acid; ASP, aspartic acid; Gly, glycine; Ser, serine; Asp, NH_2-asparagine.


MAXIMUM DENSITY OF SPOT
LEU
VAL
γ-NH2-BUT
TYR
UN-17
β-ALA
α-ALA
0 20 60 100 140 180 220
HOURS AFTER DETACHMENT


MAXIMUM DENSITY OF SPOT
GLU
ASP
GLY
SER
ASP-NH2
5 20 60 100 140 180 220
HOURS AFTER DETACHMENT


MAXIMUM DENSITY OF SPOT
LEU
VAL
γ-NH2-BUT
TYR
β-ALA
α-ALA
20 60 100 140 180 220 260
HOURS AFTER DETACHMENT


MAXIMUM DENSITY OF SPOT
GLU
ASP
GLY
SER
20 60 100 140 180 220 260
HOURS AFTER DETACHMENT

Figures 1 to 5 show the behavior of nonprotein free amino acids and amides during excised leaf culture in continuous light and dark and in the leaves exposed to light after an initial long period of darkness. While the free amino acids accumulated both in light and dark, the content of leucines, valine, γ-aminobutyric acid, tyrosine, UN-17, and serine maintained higher levels up to the late hours in light as compared to the dark, showing almost comparable amounts in the initial period (figs 1 and 2). During late hours in dark these amino acids show a decrease. In contrast to these, glutamic and aspartic acids exhibit very low levels during the initial hours in light as compared to their massive accumulation in the dark (fig 2). Glycine, which showed a constant increase up to the late hours of culture in the dark, also gave lower values in light. This may be due to the active participation of these amino acids in the synthesis of protein in detached leaves in light which has been recently well supported (21, 28). The slow increase in the content of these two acids toward the end of the experiment even in light is explainable in view of the finding of Racusen and Aronoff (21) who showed that with increasing time after excision of the leaf from the plant the capacity for protein synthesis is greatly reduced. Thus the acids which contribute to the synthesis of protein slowly accumulate. The sharp increase in the content of glutamic acid, aspartic acid, glycine, and to some extent in other amino acids (figs 3 and 4) in the leaves exposed to light after an initial dark period also confirms this suggestion. However, the small increases in other amino acids like leucine, phenylalanine, valine, and at late hours after transfer in γ-aminobutyric acid might also be partly due to sparring action of carbohydrates for the utilization of these amino acids in the energy reactions of the leaf, which it does in the leaves in dark.

Contrary to the earlier report (23, 24) α-alanine did not show any marked accumulation in S-20 variety of tobacco leaves although it has been found in amounts greater than aspartic and glutamic acids in the protein. This may indicate immediate oxidation of this acid, or its utilization in some other transformations, immediately upon its liberation. However, this difference appears to be distinctly varietal since cheroot variety and the two varieties of *Nicotiana rustica* (unpubl.) showed accumulation of alanine during the early hours in dark followed by a decrease in the late hours as has been reported earlier.

Of the two amides, glutamine and asparagine, only asparagine accumulated in both light and in the dark, the amounts formed in light were always lower as compared to the dark. Glutamine could not be found in detectable quantities even in light; instead proline accumulated in massive amounts (fig 5). γ-aminobutyric acid too showed a steady increase in light whereas in dark a decrease was observed after an initial accumulation up to 75 hours. Mothes (1956) also cites examples where proline and γ-aminobutyric acid are known to substitute for glutamine.

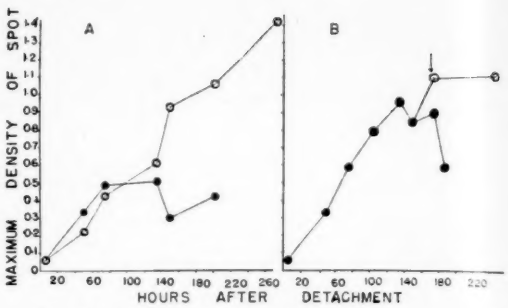


FIG. 5A. Behavior of proline in tobacco leaves cultured in water in light and in dark, and (B) in the leaves exposed to light after an initial dark period. Open circles, light; closed circles, dark.

SUGARS: From figure 6 it is clear that glucose and fructose accumulate in considerable amounts during the first 75 hours in the dark, after which they decrease. However, the increase in the sucrose content up to 105 hours of culture in the dark presents an interesting feature, since sucrose is quickly depleted during the starvation period in the majority of leaf species worked out. As the idea of direct formation of sucrose from starch in darkened leaves has been ruled out by Meeuse (19) on the grounds of their molecular structures, its increase can be explained if

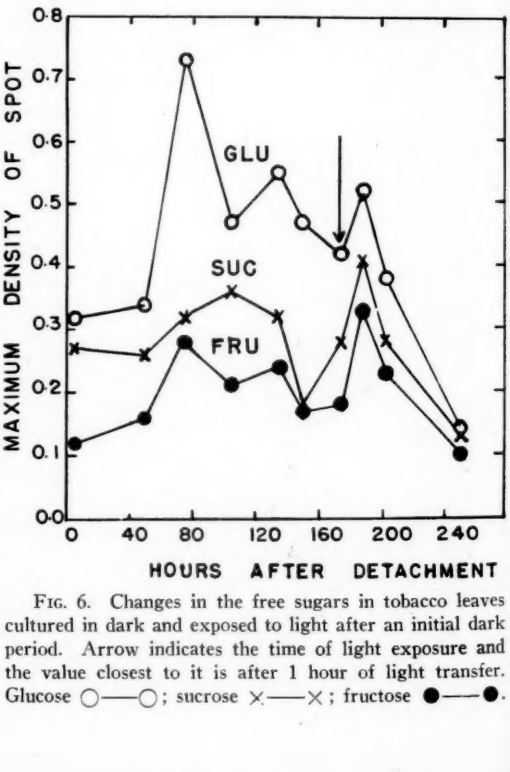


FIG. 6. Changes in the free sugars in tobacco leaves cultured in dark and exposed to light after an initial dark period. Arrow indicates the time of light exposure and the value closest to it is after 1 hour of light transfer. Glucose ○—○; sucrose ×—×; fructose ●—●.

a sucrose-phosphorylase (13) is considered operative in Nicotiana leaves. Recently, Vittorio, Krotkov, and Reed (39) have demonstrated an enzyme which catalyzes the synthesis of sucrose from glucose and glucose-1-phosphate, although it has not been shown to be identical to the sucrose-phosphorylase of bacteria. The increase of sucrose in the leaves exposed to light after an initial dark period, prior to any increases of glucose and fructose, as well as the increase of both glucose and fructose along with sucrose during the next 12 hours are also consistent with the findings of Vittorio et al (39). Unpublished results from this laboratory (Laloraya 1958) have shown that the two varieties of *Nicotiana rustica* L. (Motihari and N.P. 18) do not show increases in the sucrose content in darkened leaves and contrary to *N. tabacum* var. S-20, their sucrose content was quickly depleted. Hence the occurrence of such enzyme systems differs in different species of the genus.

Organic Acids: In the numerous experiments carried out with *Nicotiana tabacum* var. S-20 leaves, an organic acid metabolism typical of tobacco leaves (33), could never be found. Malic acid was the only dominating acid in these leaves and citric, succinic, and oxalic acids were present only in traces. During culture in both dark and light all the acids maintained their respective levels and exposure to light after an initial dark period also caused no noticeable effect. The malic-citric conversion leading to the accumulation of citric acid and depletion of malic acid, in the dark could not be observed. Two varieties of *Nicotiana rustica* L. (N.P. 18 and Motihari) have also shown similar results (unpubl.). Chibnall (6) also cites some cases on record where citric acid did not accumulate during the first 100 hours of starvation (p 242).

Behavior of Nicotiana Tabacum var. Cheroot Leaves Cultured in Distilled Water and in Streptomycin Penicillin Solution in Dark: Table I summarizes the results of the chromatographic analyses of organic acids in cheroot leaves as deduced from the relative changes in spot area and intensity on the chromatograms. It will be seen that in the leaves cultured in distilled water in the dark malic acid decreased while citric acid correspondingly increased during the first 72 hours of culture. At 125 hours there was no further decrease in malic acid or increase in citric acid. In the leaves cultured in antibiotic solutions, however, citric acid did not accumulate and malic acid retained its original level throughout the culture period. Succinic acid and the unidentified acid (band No. 4) too offered no changes.

The changes in the free amino acid and amide content as deduced from the density changes of the spots on the chromatograms, are shown in table II.

Although the general metabolic trend as regards the amino acid metabolism is not at variance with the earlier findings, yet there are significant differences associated with the Cheroot variety leaves. Whether these changes are varietal, or partly due to the change in the working season, is not known, since a systematic study of the changes in the amino acid metabolism in different seasons has not been attempted. A new range of free amino acids, viz., leucine and isoleucine, phenylalanine, valine, tyrosine, threonine, histidine, β-alanine, cysteic acid, and the unidentified substances 2 and 16 appeared only after 48 hours of detachment and culture on water as well as on antibiotic solution. Increases in other amino acids, which were initially present in the fresh leaves viz., γ-aminobutyric acid, α-alanine, glutamic acid, aspartic acid, glycine and serine, and arginine were also observed. The unidentified positive ninhydrin reacting substance (11) which appeared prominently on the chromatograms after 48 hours of culture in both water and antibiotic solution showed a decrease during the next 24 hours so much that none of it

Table I

Changes in Content of Organic Acids in Nicotiana Tabacum Var. Cheroot Leaves

Organic Acid	Expt	Hrs in dark			
		Initial sample	48	75	125
Succinic	D.W.	1	1	1	1
	S.P.	1	1	1	1
Malic	D.W.	6	5	2	2
	S.P.	6	6	6	6
Citric	D.W.	2	3	6	5
	S.P.	2	2	2	2
Unidentified	D.W.	2	2	2	2
	S.P.	2	2	2	2
Oxalic	D.W.	1	1	1	1
	S.P.	1	1	1	1

D.W., distilled water; S.P., streptomycin + penicillin solution.

TABLE II

CHANGES IN NON-PROTEIN AMINO ACIDS AND AMIDES IN NICOTIANA TABACUM VAR. CHEROOT LEAVES

AMINO ACIDS	INITIAL SAMPLE	HRS AFTER DETACHMENT AND CULTURE IN DARK					
		48		72		125	
		D.W.	S.P.	D.W.	S.P.	D.W.	S.P.
Leucine and isoleucine	...	2	2	3	3	5	4
Penylalanine	...	2	2	2	2	3	3
Unidentified I	...	...	...	1	...	3	...
Valine	...	2	2	2	2	3	3
γ-Aminobutyric	1	2	2	3	3	4	4
Tyrosine	...	2	2	2	2	3	3
Proline	1	2	2	1	1	1	1
β-Alanine	...	1	...	2	2	2	2
Unidentified II	...	3	2	...	...	...	...
α-Alanine	1	2	2	3	3	5	2
Glutamic acid	2	4	3	5	5	3	2
Glutamine	1	2	2	2	2	3	2
Aspartic acid	2	4	4	6	5	5	4
Asparagine	...	2	2	5	3	8	5
Arginine	1	2	2	4	3	4	3
Glycine	1	2	2	3	4	5	4
Serine	1	2	2	2	3	4	3
Threonine	...	1	1	2	2	1	1
Histidine	...	1	1	2	3	2	2
Lysine	...	1	...	...	...	...	...

Figures represent the relative changes in the visible density of the spots; numerals show their presence and — absence. D.W., water; S.P., streptomycin and penicillin solution.

was traceable subsequently. It may be noted that a substance having identical position to this was not detected in S-20 variety of tobacco leaves growing under similar conditions. The other unidentified substance (1) which has been detected in water cultured leaves at 72 hours and in increased quantities at 125 hours, was not traceable in the leaves cultured on antibiotic solutions.

Of particular interest has been the behavior of the amide asparagine and the amino acid alanine. Asparagine accumulated in the leaves cultured in water as well as in the antibiotic solution, the amount formed at 125 hours of culture in water being much more than in that cultured in antibiotic solution. α-alanine behaved somewhat differently than other amino acids in that its content increased progressively up to 125 hours in the leaves cultured on water, where it gave an intense spot, while those cultured on antibiotic solution showed a decrease at 125 hours after initial accumulation until 72 hours.

TABLE III

CHANGES IN CONTENT OF MALIC AND CITRIC ACIDS IN NICOTIANA TABACUM VAR. S-20 LEAVES CULTURED IN DARK

EXPERIMENT TREATMENT		INITIAL SAMPLE	HRS IN DARK			
			48	72	120	155
Distilled water	Malic	6	6	6	6	6
	Citric	1	1	1	1	1
Streptomycin and penicillin	Malic	6	6	6	6	6
	Citric	1	1	1	1	1
Na-Succinate	Malic	6	10	12	14	14
0.05 M pH 7.5	Citric	1	2	3	8	8
K-Citrate	Malate	6	12	12	10	10
0.05 M pH 7.8	Citrate	1	6	7	12	14

BEHAVIOR OF DETACHED NICOTIANA TABACUM VAR. S-20 LEAVES GROWN IN PLOTS OF BOTANICAL GARDEN WITH ORDINARY MANURE: *Effect of culturing leaves in streptomycin and penicillin solution and in organic acid salt solutions in dark. Changes in individual organic acids*: From table III, which summarizes the results of the chromatographic analyses, note that the organic acids of the S-20 variety of tobacco leaves behaved identically in the leaves cultured in water and in those in antibiotic solution. In neither case was malic converted to citric acid. Malic acid kept its constant level and succinic, citric, and oxalic acids also remained unchanged and were present only in small amounts.

Organic acid feeding experiments clearly show that succinate and citrate salts when fed to these leaves were metabolized and converted to other acids of the citric acid cycle. The leaves cultured on succinate solution showed large increases of malic acid even during the first 48 hours of dark culture and citric acid also appeared in concentrations much above the control. Both malic and citric acid showed progressive increases up to 150 hours. Free succinic acid had not been located in detectable amounts on the chromatograms, suggesting that all the succinate taken up was almost immediately metabolized to malate and citrate, due probably, to the operation of an active succinic dehydrogenase system. Citrate when fed to the leaves also resulted in marked increases in malic acid content during the first 48 hours. Citric acid itself accumulated in considerable amounts and the increase was progressive up to 155 hours. After 48 hours the level of malic acid gradually declined even in the presence of excess citrate.

BEHAVIOR OF FREE AMINO ACIDS AND AMIDES: Table IV shows the changes in the free amino acids and amides in the leaves cultured in water and in the antibiotic solution.

From the table it may be observed that the behavior of free amino acids has been identical in the leaves cultured in water and in antibiotic solution; and the general metabolic trend presented a picture similar to the earlier findings, but for the interesting observation that glutamine accumulated as much as asparagine in both sets of leaves during the culture period in dark.

There was a very low level of free amino acids in the fresh leaves, only very faint spots being detectable for γ-aminobutyric acid, proline, α-alanine, glutamic acid, aspartic acid, serine and glycine, and glutamine. Even during the starvation period the amounts of free amino acids were not comparable to those in the earlier experiments and it may be noted that whereas phenylalanine was detectable in the free state after 48 hours of detachment and culture in the dark, leucines did not appear in detectable quantities until 72 hours. Leucines were not observed at all during later hours while phenylalanine, valine, and tyrosine were present in detectable quantities up to 150 hours of starvation, after which, they too, were no longer detected. γ-aminobutyric acid, α-alanine, glutamic

TABLE IV

CHANGES IN FREE AMINO ACIDS AND AMIDES IN DETACHED NICOTIANA TABACUM VAR. S-20 LEAVES

AMINO ACIDS	CULTURE PERIOD IN HRS						MAXIMUM DENSITY OF SPOT			
	0	48		72		120		150		200	
		D.W.**	S.P.**	D.W.	S.P.	D.W.	S.P.	D.W.	S.P.	D.W.	S.P.
*Leucine and isoleucine	−	−	−	+	+	−	−	+	−	−	−
*Phenylalanine	−	+	+	+	+	+	+	+	+	−	−
*Valine	−	+	+	+	+	+	+	0.09	0.05	0.08	0.12
γ-Aminobutyric acid	0.06	0.08	0.11	0.23	0.24	0.11	0.11	+	+	−	−
*Tyrosine	−	+	+	+	+	+	+	−	−	−	−
*Proline	−	−	−	+	+	+	+	+	+	+	+
*β-Alanine	−	−	−	−	−	−	+	0.13	0.08	0.04	0.06
α-Alanine	0.09	0.09	0.10	0.19	0.15	0.10	0.10	0.28	0.14	0.14	0.16
Glutamic acid	0.20	0.20	0.33	0.30	0.34	0.22	0.16	0.30	0.35	0.28	0.34
Glutamine	0.13	0.15	0.21	0.32	0.14	0.26	0.30	0.08	0.05	0.05	0.05
Aspartic acid	0.11	0.12	0.15	0.15	0.24	0.10	0.06	0.18	0.20	0.28	0.26
Asparagine	−	0.10	0.10	0.10	0.08	0.13	0.21	0.23	0.12	0.17	0.20
Serine and glycine	0.05	0.13	0.23	0.23	0.25	0.14?	0.28	+	+	+	+
*Threonine	−	+	+	+	+	+	+	−	−	−	−

* Density values were not obtained for the amino acids which were either present as faint though distinct spots or which appeared only at certain periods of the culture. In such cases a + sign has been put to show its presence and a − its absence.

** D.W., distilled water; S.P., streptomycin + penicillin solution.

acid, and aspartic acid, however, showed initial increases up to 72 hours, whereafter they decreased, although they remained present in detectable quantities. Serine and glycine exhibited increases up to 150 hours and decreased at 202 hours. Threonine, histidine, arginine, and proline were detected only as very faint spots during the culture period; proline was traceable only at 72 hours and 125 hours of culture.

Discussion

Two important facts seem to emerge from the experiments described, the first regarding the behavior of organic acids in different varieties and the second dealing with the regulation of glutamine level in detached leaves. The lack of malic-citric conversion in variety S-20 of tobacco leaves and in two varieties of *Nicotiana rustica* (N.P. 18 and Motihari) (unpubl.)and its presence in Cheroot and Connecticut tobacco would suggest that the particular enzyme responsible for malic-citric conversion in detached darkened leaves may not be operative in certain varieties of tobacco, or in other words it may be said that certain varieties possess enzyme systems which may not be of general occurrence in plants. This will reflect on the genic control of such enzyme systems (14).

The suppression of malic-citric conversion in the presence of antibiotic solution in Cheroot tobacco leaves, and the fact that antibiotic culture does not alter the behavior of organic acids in leaves of the S-20 variety, where the results are consistent with the operation of a normal citric acid cycle, suggests that the peculiar conversion of malic acid to citric acid in certain varieties of tobacco leaves, during culture in dark, might be due to some secondary reaction not directly associated with the vital oxidative cycle operative in the tissue. Any blockade by streptomycin and penicillin in the normal oxidative cycle would have resulted in the accumulation of end product at the blockade site, leading ultimately to the death of the tissue in the S-20 as well as the Cheroot variety of leaves where the organic acids maintained a constant level in accord with the operation of a normal cycle. Succinate and citrate feeding experiments clearly show that in the S-20 variety the enzyme systems responsible for the citric acid cycle were operative, succinic dehydrogenase being most active, and the pathway from malic to citric acid evidently existed. The accumulation of free citric acid in the leaves fed with citrate and succinate is due partly to sluggish conversion of citric acid itself to other acids in Nicotiana leaves (44) and partly to the pH 7.8 of the culture solution since Vickery and Hargreaves (34) have shown that citrate ion was taken up more readily from culture solution at pH 4 but accumulated more in the tissue from solutions at pH 7.

The inhibition of the malic-citric conversion by the antibiotic raises the important question as to what could be the nature of the enzymatic system operative in these leaves which could be blocked by streptomycin and penicillin. Work on bacteria *E. coli*, (31) has shown that streptomycin blocks the oxalacetate-acetyl CoA condensation enzyme leading to the death of streptomycin sensitive cells and that the resistant cells do not possess the oxalacetate-acetyl CoA condensation enzyme. However, work with higher plants does not record any such blockade of enzyme system, nor is such a possibility borne out by the experiments described. Recently Rosen (26) and Gray (11) have independently shown that streptomycin affects the normal metabolism of plants and is capable of inhibiting root growth and plant growth. That this inhibition is reversed by adding manganese has also been shown, and evidence has been put forward to indicate streptomycin-manganese complex formation (27). Manganese is known as the prosthetic group of several enzymes; special mention may be made of malic enzyme, the decarboxylases and the isocitric dehydrogenase.

Considering that the conversion may not be due to the enzymes of the regular citric acid cycle the only other known pathway which would bring about this conversion seems to be in the operation of malic enzyme (20). Malic enzyme has been recently recorded in plant tissues (1, 2, 32, 37). However, evidence for the occurrence of other alternative pathways has also been recently reported (15).

Vickery et al (1937) have found that approximately two molecules of malic acid were being consumed for the synthesis of one molecule of citric acid; they suggested that citric acid might have arisen in the following sequence:

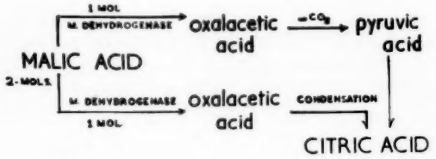


Fig. 7.

Chibnall, however, pointed out that if a malic acid-citric acid transformation of the type Vickery et al suggest was operative, and malic acid was not being utilized for other metabolic purposes such as amide production, then the malic acid loss to citric acid gain should have been approximately constant throughout, which, however, was not found to be so. Chibnall suggested the following alternative pathway:

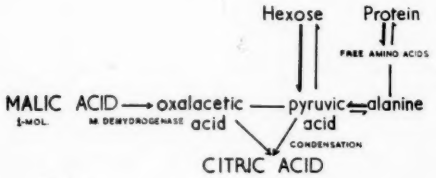


Fig. 8.

The formation of a greater amount of asparagine and increase in alanine up to 125 hours in water-cultured leaves as compared to those cultured in antibiotic solution indicates that C-residues formed during malic acid-citric acid transformation were available in excess for the synthesis of asparagine and alanine. The increase in the content of alanine in water-cultured leaves can be explained only if it is assumed that metabolic events proceeded as suggested by Vickery et al (33) and that more pyruvic acid was being generated in the water-cultured leaves, both through the glycolytic pathway as well as from malic acid, and that the pyruvic acid so formed underwent transamination to yield alanine and was partly degraded to acetyl CoA which in its turn could condense with oxalacetate to produce citric acid. The decrease in the alanine content in the antibiotic cultured leaves at 125 hours may be taken to indicate the normal course of events where normal glycolytic pathway and citric acid cycle, both were operative. However, the course proposed from malic acid to pyruvic acid does not seem to pass through normal citric acid cycle enzymes, as suggested by Vickery et al (33) and the conception of malic enzyme operative in these leaves meets Chibnall's criticism as well as the results of Vickery et al. The inhibition of this conversion by streptomycin and penicillin may be due to the chelation of the Mn ion of the malic enzyme, with the streptomycin (27) thus making it inactive. The following scheme is a tentative pathway that explains the metabolic relationship during malic-citric acid conversion:

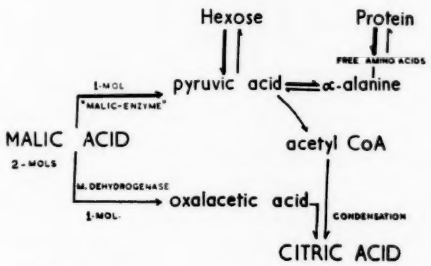


FIG. 9.

The absence of amide glutamine in the first series of experiments from the S-20 variety of tobacco leaves cultured in both light and dark and a simultaneous accumulation of proline in these leaves together with the fact that glutamine accumulated as much as asparagine even in dark with only traces of proline, if at all present, in the other set of experiments, from plants grown under different soil conditions, strongly suggests that glutamine level in isolated leaves would depend on other factors rather than on light and dark or the carbohydrate status of the tissue, and that probably there exists a system for interconversion between the two components. This, indeed, has been found to be so (8, 29). Glutamine synthetase has been isolated and purified from plant tissues (9, 40) and its existence cannot be doubted.

The overall metabolism of detached Nicotiana leaves may be summarized thus:

In the first 72 hours in dark, starch was being quickly depleted for the energy requirements of the starving leaves. Hexoses and sucrose accumulated during this period. However, rapid breakdown of protein was simultaneously occurring. The early massive increases of the two important acids (aspartic and glutamic) could be explained since excess of oxalacetate and α-ketoglutarate links are made available through the rapid hydrolysis of starch for the synthesis of these two acids apart from that derived by protein breakdown. Increases in the other amino acids were largely due to protein breakdown and possible interconversions within the wide range of amino acids.

From 70 hours onwards the carbohydrates were no more sufficient, and the accumulated hexoses and sucrose were now being used into the respiratory cycle along with the free amino acids, especially the glutamic and aspartic acids and to less extent other amino acids. This represents the protein phase as suggested by Chibnall. Simultaneously in Cheroot variety, malic acid was being quickly depleted resulting into the accumulation of citric acid; the C-residues during this conversion were also available for the formation of asparagine and alanine. In variety S-20, however, the respective levels of malic acid and citric acid remained unchanged consistent with the idea of a normal citric acid cycle being operative. The interpolation of an organic acid phase does not seem to be justified since the phenomenon of malic-citric conversion is not of general occurrence in tobacco leaves and is not vital for the energy requirements of the starving leaf.

From 120 to 150 hours, more and more of the amino acids were contributing to the regulation of the cycle and thus leucines, valine, α-alanine, proline, γ-aminobutyric acid, and glycine and serine all showed a decreasing level during these late hours. Amino acids are known to yield members to the Krebs citric acid cycle directly through glutamic acid, aspartic acid and alanine, and indirectly through proline, histidine, arginine, leucine, and others.

In the last phase, when the leaves started browning, and exhibited signs of death they probably lost all their power of selectivity and even organic acids were sometimes found to be depleted. Thus extreme depletion of the most of the free amino acids and probably, to some extent, of asparagine and malic acid represents this stage.

The data agree well with the findings of Yemm (42) with barley leaves and support his conclusions regarding the utilization of amino acids, obtained by degradation of protein, for carrying out the energy reactions of the starving leaf.

In light, however, as carbohydrates were always abundant in the leaves, the protein breakdown was simultaneously occurring, the free amino acids accumulated as much as in the dark, but the respective

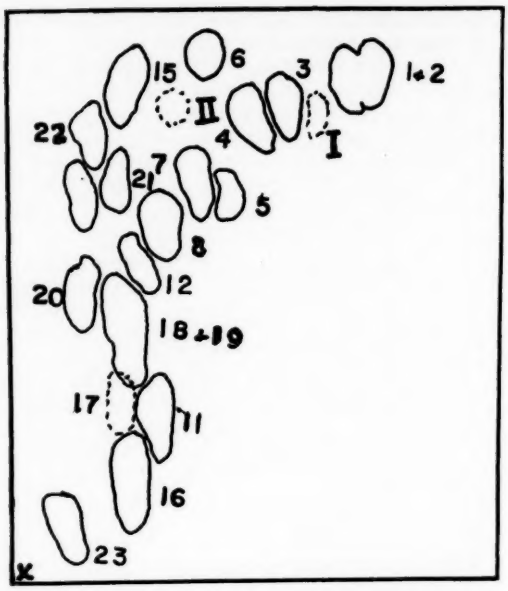


FIG. 10. Diagram of a two dimensional chromatogram showing the positions of various amino acids present in two varieties of *Nicotiana tabacum*: 1 and 2, leucines and phenylalanine; 3, valine; 4, γ-aminobutyric acid; 5, tyrosine; 6, proline; 7, β-alanine; 8, α-alanine; 11, glutamic acid; 12, threonine; 15, arginine; 16, aspartic acid; 18 and 19, serine and glysine; 20, asparagine; 21, glutamine; 22, histidine and lysine; 23, cysteic acid. Unidentified substances shown by dotted circles, 1, 11, and 17. Solvents used were: phenol: ammonia 0.5 %: water in the vertical direction and butanol: acetic acid: water (4:1:5) in the horizontal direction.

levels of glutamic acid, aspartic acid, and of glycine were always lower during the initial hours than in the dark possibly due to their utilization in the synthesis of protein which proceeds simultaneous to the breakdown in excised leaves cultured in the light at a more rapid rate as compared to in the dark. The high level of various other free amino acids up to late hours in light seems to be due to the sparing action of carbohydrates in relation to free amino acids. However, the decrease in the content of most of the free amino acids during late hours even in light could be due to the deteriorating condition of the leaf during its long period of excision.

SUMMARY

Changes in the protein, soluble nitrogenous compounds, free sugars, and organic acids have been studied in two varieties of tobacco leaves cultured in distilled water in light and dark and in streptomycin and penicillin solution, using paper chromatographic methods. Evidence is presented that although the total amino-nitrogen content increases both in light and dark in detached leaves, the behavior of individual amino acids varies considerably. Evidently non-protein amino acids and amides in detached leaves undergo interconversions within themselves and these interconversions are probably regulated by certain growth factors.

Behavior of free sugars and organic acids has also been studied in an attempt to give an overall picture of metabolic events operative in leaves cultured in light and dark, after removal from the plant. S-20 variety of tobacco leaves failed to show any malic-citric conversion when cultured in water in dark. Cheroot variety, however, showed typical malic acid-citric acid conversion as has been reported for Connecticut tobacco (33) but they failed to do so when cultured in antibiotic solution. Culture of S-20 variety leaves in antibiotic solution did not result in any alteration of the behavior of organic acids. Feeding experiments have shown that the enzymes of regular citric acid cycle are operative in this variety. It is suggested that the peculiar behavior of organic acids in certain varieties of tobacco leaves is due to the operation of enzyme systems other than those encountered in the Krebs citric acid cycle, probably malic-enzyme, and that this is blocked in the presence of antibiotics.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. ANDERSON, I. and H. J. EVANS. 1956. Effect of manganese and certain other metal cations on isocitric dehydrogenase and malic enzyme activities in *Phaseolus vulgaris*. Plant Physiol. 31: 22–28.
2. ARNON, D. I. 1951. Extracellular photosynthetic reactions. Nature 167: 1008–1010.
3. BLOCK, R. J., E. L. DURRUM, and G. ZWEIG. 1956. A Manual of Paper Chromatography and Paper Electrophoresis. P. Academic Press, N. Y.
4. BUCHAN, J. L. and R. I. SAVAGE. 1952. Paper chromatography of some starch conversion products. Analyst 77: 401–406.
5. BULL, H. B., J. W. HAHN, and V. R. BAPTIST. 1949. Filter paper chromatography. Jour. Amer. Chem. Soc. 71: 550–553.
6. CHIBNALL, A. C. 1939. Protein Metabolism in Plants. Yale University Press, New Haven.
7. CONSDON, R., A. H. GORDON, and A. J. P. MARTIN. 1944. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. Jour. 38: 224–232.
8. DAVIS, B. D. 1955. Intermediates in amino acid biosynthesis. Advances in Enzymol. 16: 247–312.
9. ELLIOTT, W. H. 1953. Isolation of glutamine synthetase and glutamotransferase from green peas. Jour. Biol. Chem. 201: 661–672.

10. Fowden, L. 1954. The nitrogen metabolism of ground nut plants: the role of γ-methylene glutamine and γ-methyleneglutamic acid. Ann. Bot. 18: 417–440.
11. Gray, R. A. 1955. Inhibition of root growth by streptomycin and reversal of the inhibition by manganese. Amer. Jour. Bot. 42: 327–331.
12. Gregory, F. G. and P. K. Sen. 1937. Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium deficiencies. Ann. Bot. 1: 521–561.
13. Hassid, W. Z. 1954. Biosynthesis of complex saccharides. In: Chemical Pathways in Metabolism. I. Pp. 521–561. Academic Press, N. Y.
14. Horowitz, N. H. 1950. Biochemical genetics of Neurospora. Advances in Genet. 3: 33–71.
15. Kornberg, H. L. and H. A. Krebs. 1957. Synthesis of cell constitutents from C_2 units by a modified tricarboxylic acid cycle. Nature 179: 988–991.
16. Krebs, H. A. 1943. The intermediary stages in the biological oxidations of carbohydrates. Advances in Enzymol. 3: 191–252.
17. Krotkov, G. 1939. Carbohydrate and respiratory metabolism in the isolated starving leaf of wheat. Plant Physiol. 14: 203–226.
18. McKee, H. S. 1951. Studies on the nitrogen metabolism of barley plant (*Hordeum sativum*). Australian Jour. Sci. Research B. Biol. Sci. 3: 474–486.
19. Meeuse, B. J. D. 1949. Observations on the enzymatic action of maple and birch saps. New Phytol. 48: 125–142.
20. Ochoa, S., A. H. Mehler, and A. Kornberg. 1948. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalysing the reversible oxidative decarboxylation of L-malic acid. Jour. Biol. Chem. 174: 979–1000.
21. Racusen, D. W., and S. Aronoff. 1954. Metabolism of soybean leaves. Exploratory studies in protein metabolism. Arch. Biochem. Biophys. 51: 68–78.
22. Ranjan, S., Govindjee, and M. M. Laloraya. 1955. Chromatographic studies on the amino acid metabolism of healthy and diseased leaves of *Croton sparsiflorus*, Morong. Proc. Nat. Inst. Sci. India. 21: 42–47.
23. Ranjan, S. and M. M. Laloraya. 1955. Amino acid metabolism of starving *Phaseolus vulgaris* leaves. Naturwiss. 42: 537–537.
24. Ranjan, S. and M. M. Laloraya. 1956. Amino acid metabolism of Nicotiana leaves after removal from the plant. Nature 177: 235–236.
25. Ranjan, S. and T. Rajarao. 1955. Separation of organic acid by circular paper chromatography: Diurnal variation in the organic acid content of Pelargonium. Naturwiss. 42: 581–581.
26. Rosen, W. G. 1954. Plant growth inhibition by streptomycin and its prevention by manganese. Proc. Soc. Exp. Biol. Med. 85: 385–388.
27. Rosen, W. G. 1955. A study of plant growth inhibition and metal complex formation by streptomycin. University of Wisconsin publication No. 14730.
28. Stephenson, M. L., K. V. Thimann, and P. C. Zamecnik. 1956. Incorporation of C^{14} amino acids into proteins of leaf discs and cell free fractions of tobacco leaves. Arch. Biochem. Biophys. 65: 194–209.
29. Steward, F. C., R. G. S. Bidwell, and E. W. Yemm. 1958. Nitrogen metabolism, respiration, and growth of cultured plant tissue. Jour. Exp. Bot. 9: 11–51.
30. Steward, F. C., R. H. Wetmore, J. F. Thompson, and J. P. Nitsch. 1954. A quantitative chromatographic study of nitrogenous components of shoot apices. Amer. Jour. Bot. 41: 123–134.
31. Umbreit, W. W. 1949. A site of action of streptomycin. Jour. Biol. Chem. 177: 703–714.
32. Vennesland, B., M. C. Gollub, and J. F. Speck. 1949. The β-carboxylases of plants. I. Some properties of oxalacetic carboxylase and its quantitative assay. Jour. Biol. Chem. 178: 301–314.
33. Vickery, H. B., G. W. Pucher, A. J. Wakeman, and C. S. Leavenworth. 1937. Chemical investigations of the tobacco plant. VI. Chemical changes that occur in leaves during culture in light and in darkness. Bull. Conn. Agr. Exp. Sta. No. 399.
34. Vickery, H. B. and C. A. Hargreaves, II. 1952. The metabolism of organic acids of tobacco leaves. IV. Effect of culture of excised leaves in solutions of citrate. Jour. Biol. Chem. 196: 409–422.
35. Vickery, H. B. and C. A. Hargreaves, II. 1952. The metabolism of organic acids of tobacco leaves. V. Effect of culture of excised leaves in solutions of succinate and L-malate. Jour. Biol. Chem. 197: 121–131.
36. Vickery, H. B. 1955. The metabolism of organic acids of tobacco leaves. VIII. Effect of culture of excised leaves in solutions of L-malate at pH 4 to pH 7. Jour. Biol. Chem. 214: 323–333.
37. Vickery, H. B. and J. K. Palmer. 1957. The metabolism of organic acid of tobacco leaves. XIII. Effect of culture of excised leaves in solutions of potassium bicarbonate. Jour. Biol. Chem. 227: 69–81.
38. Viets, F. G., E. I. Whitehead, and A. L. Moxon. 1947. Nitrogen metabolism of detached corn leaves in darkness and in light. Plant Physiol. 22: 465–476.
39. Vittorio, P. V., G. Krotkov, and G. B. Reed. 1954. Synthesis of radioactive sucrose by tobacco leaves from C^{14} uniformly labeled glucose and glucose-1-phosphate. Can. Jour. Bot. 32: 369–377.
40. Webster, G. C. 1953. Enzymatic synthesis of glutamine in higher plants. Plant Physiol. 28: 724–727.
41. Wood, J. G. and D. H. Cruickshank. 1944. The metabolism of starving leaves. V. Changes in amount of some amino acids during starvation of grass leaves, and their bearing on the nature of relationship between proteins and amino acids. Australian Jour. Exp. Biol. Med. Sci. 22: 111–123.
42. Yemm, E. W. 1937. Respiration of barley plants. III. Protein catabolism in starving leaves. Proc. Roy. Soc. (London) B. 123: 243–273.
43. Yemm, E. W. 1949. Glutamine in the metabolism of barley plants. New Phytol. 48: 315–331.
44. Zbinovsky, V. and R. H. Burris. 1952. Metabolism of infiltrated organic acids by tobacco leaves. Plant Physiol. 27: 240–250.

FREE AMINO ACIDS IN ALFALFA AS RELATED TO COLD HARDINESS[1, 2]

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Alfalfa, *Medicago sativa* L., is one of the most widely grown legumes in the northern areas of the United States and Canada. It provides an economical source of protein and other nutrients to all classes of livestock. Many of the highly productive varieties are either killed or severely weakened by winter conditions in the northern areas. A better understanding of the physiology and biochemistry of cold resistance would aid in the improvement and maintenance of this crop in the north.

Many investigations have been made in an attempt to correlate or associate some component of the alfalfa plant with the development of winter hardiness. Although no direct correlations have been established, some investigators (1, 11, 12, 13) have suggested that protein and other nitrogenous components of the plant may be responsible in part for winter hardiness.

In early work Rosa (8) reported that the hardening process in plants is accompanied by a marked increase in water-retaining power of the cell colloids of the plant. In more recent investigations a higher soluble nitrogen content was reported in hardy alfalfa roots compared to non-hardy roots (3, 6, 18).

Investigations on the biochemistry of frost resistance have been hindered because it has been established only recently that frost resistance is largely a property of the protoplasm of living cells. These advances have come about largely through the work of Scarth and coworkers (4, 5, 10, 14, 15). Scarth (9) points out that it seems to be the rule that extreme resistance to cold, whether in plant or animal, is associated with the ability of cells to endure desiccation. His studies suggest a greater water binding capacity of hardy cell colloids. The intimate association of proteins with the cell, either as components of enzyme systems or in structural organization, has suggested that changes in these constituents may be involved in the development of cold hardiness.

Siminovitch and Briggs (11, 13) working with the live bark of the Black Locust tree in relation to frost hardiness, found among the several nitrogen fractions studied that the increase in the water-soluble proteins was correlated positively with the development of hardiness in the fall. Subsequently these proteins decreased in the spring as hardiness disappeared. Further evidence for an association between hardiness and soluble protein was gained by removing the top of the tree from the stump which had developed hardiness. In the spring, the seasonal decrease in protein concentration from the hardened bark of the tree was retarded at the same rate as dehardening of the tree stump compared to the change observed in normal trees. In alfalfa, Bula and Smith (2) found a high correlation between the trends of soluble protein nitrogen and cold resistance in hardy varieties at both Palmer, Alaska, and at Madison, Wis. No such relationship was found in the non-hardy variety studied.

Briggs and Siminovitch (1) in further studies with the live bark of the Black Locust tree observed five electrophoretically distinguishable components among the soluble proteins. There was a marked shift in the pattern but not in the number of components between midsummer and winter proteins. They emphasized Scarth's (9) observation that the most characteristic physical property of frost hardy cells was their capacity to withstand injury by dehydration (12). To date, there has been no report in the literature involving a quantitative analysis of free amino acids in relation to hardiness. This paper deals with a study of the free amino acids in alfalfa varieties as hardiness developed, and the relationship of these amino compounds to the soluble protein fraction and hardiness.

MATERIALS AND METHODS

Three varieties of alfalfa, Vernal (very winter hardy), Buffalo (intermediately hardy), and Cali-

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verde (non-hardy) were seeded in rows 2 feet apart during mid-May at Madison, Wis., in 1957 and 1958. Cloned plantings were made of both varieties in 1957 to obtain genetically uniform material. The plants were weeded, sprayed for insects, and cared for as needed through each growing season. In each year, a random sampling of approximately 50 seedling roots from each variety was made in mid-August, mid-October, and mid-December. However, prior to the December harvest, the plants were dug from the soil in late November before soil freezing and grouped together in clay pots (surrounded by soil) and placed back in the ground at the soil surface.

The harvested intact plants were washed free of soil. The 4-inch section of the root just below the crown from ten plants of each variety was frozen immediately in liquid air to prevent enzymatic change. The roots were ground by passing the frozen sections first through a common food grinder along with dry ice and next through a 20-mesh sieve of a Wiley mill. The mill and grinder were precooled with dry ice and the particles to be passed through were mixed 1:1 with dry ice to maintain a very brittle condition. This finely divided material was stored at −20° C until lyophilization was accomplished (usually within 1–2 days).

The frozen samples were lyophilized on a watch glass in a 250-centimeter vacuum desiccator over phosphorus pentoxide. The sample was mixed 1:1 with dry ice and placed in a desiccator equipped with a cold trap in a deep-freeze at −20° C. After 3 to 4 hours of lyophilizing, the deep-freeze was brought to room temperature. The dried samples were weighed and placed in manila envelopes for storage under nitrogen in a desiccator containing phosphorus pentoxide.

Six hundred mg of each sample were placed in a 250 ml round bottom flask (ground glass joint) and refluxed for 4 hours at 60° C with 100 ml of acetone-water (1:1). Following the extraction period, each sample was filtered and the residue washed with 50 ml of acetone-water (1:1). By means of a rotating vacuum film-evaporator, the extracts were concentrated to dryness. After concentration, exactly 3 ml of the acetone-water mixture and a few glass beads were added, and after a uniform mixture was obtained, the sample was centrifuged for 15 minutes at 14,000 × G. The supernatant was removed and stored in the deep-freeze.

All chromatograms were run ascending on 28.5 by 28.5 cm. Whatman No. 1 chromatographic paper at 20° C. A volume of 20 lambda was spotted in one corner of the paper, 4 cm from the edge. The paper was tied with thread to form a cylinder, equilibrated with the solvent in a 6 × 18 inch cylindrical chamber and placed in a 5-inch crystallizing dish containing 5 ml of the solvent. The first solvent system consisted of secondary butanol: formic acid: water (75:12:12). After the solvent front had moved to the top of the paper, the paper was removed and dried in a hood. Then it was chromatographed in the second direction using a citrate buffered phenol solvent[4]. A special technique was employed in using this solvent system. Inside the 5-inch crystallizing dish holding the solvent was placed a small 50-ml beaker containing a sodium cyanide[5] solution. The hydrogen cyanide produced by the acidic phenol atmosphere binds the heavy metals on the paper which tend to oxidize the phenol. Oxidized phenol gives poor resolution. After the solvent had reached the top, the paper was removed and dried 5 minutes with forced air before drying more completely overnight in the hood. The dried paper was sprayed with a 0.2 % solution of ninhydrin in butanol[6]. After maximum color had developed (18 hrs at room temp.), an estimation of the intensity of each amino acid spot was made by rating from zero to four. Known standards were run in the same system to identify the spots. Photographs were made of the paper chromatograms using a red filter to intensify the blue colored spots.

The organic acids were run in the direction of the grain of the paper using ten lambda of the extracts per spot and ethyl acetate-formic acid-water (100:20:30)[7]. After solvent development the paper was dried in the hood overnight and sprayed with a mixture of aniline (2 ml), glucose (2 g), *n*-butanol (60 ml), 95 % ethanol, (20 ml), and water (20 ml) with heating for 15 minutes at 105° C.

The sugars were chromatographed in the same phenol solvent as used for the amino acids. The reducing sugars were detected by spraying with aniline phthlate[8] and developing for 15 minutes at 105° C. The non-reducing sugars were sprayed with a naphthoresorcinol reagent[9] and heated for 5 to 10 minutes at 105° C.

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[4] This was prepared by making a 6.3 % sodium citrate and 3.7 % KH_2PO_4 buffer. An excess (450 g) of phenol (Mallinckrodt containing a stabilizer) was added per liter of buffer in a separatory funnel. This was allowed to remain overnight at a temperature slightly lower than the temperature at which the chromatogram was run. The upper phenol rich layer was separated and centrifuged for 5 minutes at 3,000 rpm. The phenol separated from the water droplets was decanted off and used within 1 week. This phenol should not be used at a temperature below that at which it was separated.

[5] It was important to replace the sodium cyanide solution when it became yellow. Care was exercised to avoid breathing the vapors when working around the chambers.

[6] One-half g of ninhydrin was mixed with 12.5 ml of 2 *N* acetic acid and made to 250 ml with normal butanol.

[7] The mixture was placed in a separatory funnel and the two phases were shaken two times a day. After 2½ to 3 days, transesterification produces ethyl formate. It was used immediately when the transesterification was complete.

[8] Phthalic acid (1.63 g) was dissolved in 100 ml of water saturated with normal butanol and then 0.93 g aniline was added.

[9] This was prepared from two stock solutions. Stock solution A consisted of 0.2 % by weight 1, 3-napthalenediol in ethanol prepared fresh weekly. Stock solution B was 2 % trichloroacetic acid in water. These were mixed 1:1 before spraying.

TABLE I

ACIDIC COMPOUNDS AND SUGARS ESTIMATED BY PAPER CHROMATOGRAPHY IN 3 ALFALFA VARIETIES*

	SEEDED PLANTINGS**									CLONED PLANTINGS**					
	CA	CO	CD	BA	BO	BD	VA	VO	VD	CA	CD	BA	BD	VA	VD
Acidic compounds															
I	1	0	0	1	0	0	1	0	0	1	0	1	0	1	0
II	0	1	1	0	1	1	0	1	1	0	1	1	1	1	1
III	2	1	0	2	1	0	2	1	0	1	0	2	0	2	0
IV	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0
V	2	0	1	3	0	1	4	2	2	3	3	0	2	0	2
Non-reducing sugars															
Sucrose	1	1	3	1	2	4	2	1	4	1	4	1	4	1	4
Reducing sugars															
Glucose	1	1	4	1	1	4	1	2	3	1	3	1	3	1	2
Fructose	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1

* Harvested at three periods as winter hardiness was developed from seeded and cloned plantings.

** The data are for the varieties Caliverde (C), non-hardy; Buffalo (B), medium hardy, and Vernal (V), very hardy, harvested in August (A), October (O), and December (D). The number indicates relative amounts estimated from size and color intensity of spots on the paper chromatogram and rated from 0 (no spots) to 1–4 (increasing amounts) making comparisons between the three varieties at a particular harvest period.

The amino acids from the 1958 root harvest were analyzed by chromatography on ion exchange resins using a Beckman-Spinco amino acid analyzer (16). After concentration of the extracts as described above, the residue was resuspended in 2 ml of the acetone-water mixture and quantitatively transferred to a 30 ml centrifuge tube and made to a final volume of 10 ml. This was centrifuged, the supernatant decanted, and the residue washed with an additional 2 ml of solvent. The resulting extract was deproteinized with picric acid according to the procedure of Stein and Moore (17) before analysis. Analysis was made according to the method described by Spackman, Stein, and Moore (16, 17).

Because of the large size of the peak containing asparagine, glutamine, and serine, an aliquot of the extracts was subjected to mild acid hydrolysis and the aspartic acid, glutamic acid, and serine again were determined. Various concentrations of acid and times of hydrolysis were studied using a standard mixture of aspartic acid, glutamic acid, serine, threonine, glutamine, and asparagine to ascertain the best method for amide hydrolysis with the least destruction of serine. It was found that 1 ml of concentrated hydrochloric acid per 10 ml of extract (approx. 1 *N*) autoclaved at 15 pounds pressure for 30 minutes gave complete hydrolysis of amides with little effect on serine or threonine. A 3 ml aliquot of the deprotenized plant extract was mixed with 0.3 ml of concentrated HCl in a glass ampule. The sealed ampule was autoclaved for 30 minutes and the contents were transferred quantitatively to a beaker and concentrated to dryness under air. The residue was redissolved in 0.02 *N* HCl and made to 3 ml. The extract was filtered and 1 ml chromatographed on the 150-cm column. Proline was used as an internal standard in quantitating the increase of aspartic and glutamic acid. The amount of the two amides was calculated from the amount of aspartic and glutamic acid originally in the extracts and that after the acid hydrolysis.

RESULTS AND DISCUSSION

Table I lists the acidic compounds and sugars in the roots of three alfalfa varieties (varying in degree of winter hardiness) sampled at three periods during the fall of 1957 from seeded plantings. The numerical ratings indicate relative amounts estimated from the size and intensity of the spots on the paper chro-

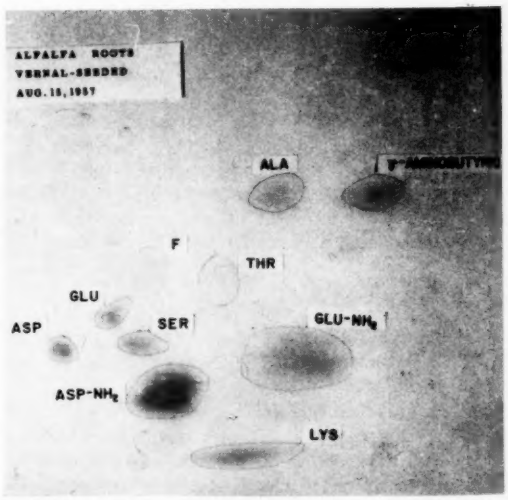

FIG. 1. Two-dimensional paper chromatogram of free amino compounds from Vernal (very hardy) alfalfa root extracts sampled in August, 1957.

TABLE II

FREE AMINO ACIDS AND OTHER NINHYDRIN-POSITIVE COMPOUNDS ESTIMATED BY PAPER CHROMATOGRAPHY IN 3 ALFALFA VARIETIES*

AMINO ACIDS	SEEDED PLANTINGS**									CLONED PLANTINGS**					
	CA	CO	CD	BA	BO	BD	VA	VO	VD	CA	CD	BA	BD	VA	VD
Glutamine***	2	2	3	1	2	4	2	2	4	2	3	2	3	2	4
Asparagine	4	1	2	1	2	2	4	2	3	3	3	3	1	2	1
Lysine	3	1	2	3	1	2	3	3	2	2	3	1	2	1	2
Serine	1	1	3	1	1	3	2	1	2	1	3	1	2	2	2
Aspartic	2	1	0	1	1	1	2	1	1	3	2	1	1	1	1
Glutamic	2	1	0	1	1	1	2	2	0	2	1	2	0	3	1
Alanine	1	1	3	1	1	3	2	1	3	1	3	1	3	2	3
α-Aminobutyric	1	1	4	1	2	3	2	1	4	2	4	2	3	2	3
Phenyl-alanine	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
Valine	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
Proline	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1
Threonine	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1
Glycine	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Leucine-isoleucine	0	0	0	0	0	0	0	0	0	1	1	1	1	2	1
B	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1
C	0	0	1	0	0	1	0	1	1	1	1	1	1	1	1
D	0	0	0	0	0	0	0	0	0	1	1	1	0	1	2
F	1	1	0	0	1	0	1	1	0	0	0	0	0	0	0
G	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1

* Harvested at three periods as winter hardiness was developed from seeded and cloned plantings.

** The data are given for the varieties Caliverde (C), non-hardy; Buffalo (B), medium hardy, and Vernal (V), very hardy, harvested in August (A), October (O), and December (D). The number indicates relative amounts estimated from size and color intensity of spots on the paper chromatogram and rated from 0 (no spot) to 1–4 (increasing amounts) making comparisons between the three varieties at a particular harvest period.

*** This spot also includes histidine and arginine.

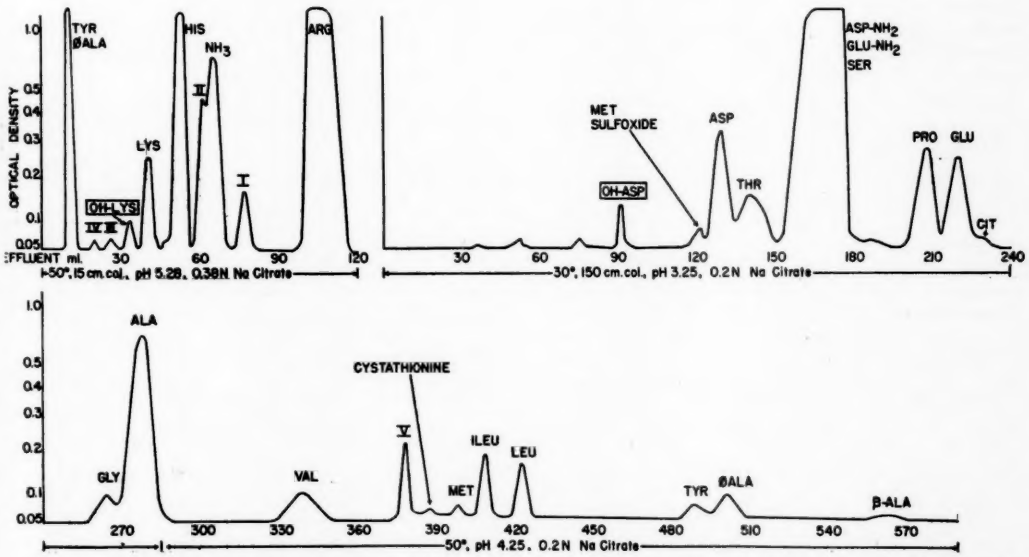

FIG. 2. A typical column chromatogram of free amino compounds from a Vernal alfalfa root extract in December, 1958. The peaks in the upper left hand corner are the basic compounds and the upper right and lower portion of the elutogram are the acidic and neutral amino compounds.

matograms. The hardy variety contained a little more of the organic acid labeled V than the non-hardy variety. There was no apparent difference in the other four acids which seemed to follow the same trends as the environmental conditions changed. The sugars were increased greatly by December in both hardy and non-hardy varieties.

Table II summarizes the estimates from the paper chromatograms of the free amino acids and other ninhydrin-positive compounds in the same samples discussed above. It was possible to observe 19 ninhydrin spots by this type of paper chromatography. Figure 1 shows a two-directional chromatogram and illustrates the separation achieved. No single component showed a pronounced difference or marked change during the sampling period. However, there were gradual shifts in the quantities of some of the components. Even though the results were not quantitative they were sufficiently interesting to justify continuing the study further. The ninhydrin reacting components were therefore measured in 195[illegible] by use of the Beckman Spinco amino acid analyzer.

Spackman, Stein, and Moore (16) have shown that the automatic recording apparatus for use in the chromatography of amino acids is capable of giving reproducible recoveries of 100 ± 3% for quantities ranging from 0.25 to 2 micromoles of amino acids. The method can give useful results with amounts as low as 1/16th micromole. The wide range of values that can be determined with close precision makes this method of amino acid analysis very useful in analyzing plant extracts where the quantity of each component may vary greatly. It was possible in the present studies to show 26 different components by column chromatography as compared to only 19 components with paper chromatography.

TABLE III

FREE AMINO ACIDS AS DETERMINED BY COLUMN CHROMATOGRAPHY ON NON-HARDY CALIVERDE (NH) AND HARDY VERNAL (H) ALFALFA ROOTS*

AMINO ACIDS	AUGUST		OCTOBER		DECEMBER	
	NH	H	NH	H	NH	H
Elution Order			*Expressed in μmoles/g dry tissue*			
Hydroxyaspartic	1.17	1.08	0.58	0.92	1.00	0.66
Methionine sulfoxide	1.42	1.00	1.25	1.08	0.92	0.75
Aspartic	10.25	10.50	29.82	14.08	5.91	5.16
Threonine	1.92	3.50	2.25	2.16	3.83	4.17
Serine	4.50	6.00	3.91	5.50	6.83	8.83
Asparagine	248.48	206.00	145.77	185.51	204.08	184.26
Glutamine	0.08	6.25	0.00	2.67	5.16	8.16
Proline	6.50	9.33	8.16	13.74	11.25	19.82
Glutamic	7.41	9.00	9.50	11.32	1.25	5.00
Glycine	1.50	2.25	1.83	2.08	2.50	2.50
Alanine	3.83	4.58	1.75	3.08	9.91	17.41
Valine	0.67	0.75	1.17	1.08	3.00	2.17
V	6.33	6.00	4.75	4.25	3.33	3.08
Cystathionine	0.00	0.00	0.00	0.00	0.25	Trace
Methionine	0.25	0.17	0.08	0.17	0.33	0.25
Isoleucine	0.25	0.33	0.83	0.75	2.17	1.58
Leucine	0.25	0.33	0.50	0.50	2.41	1.50
Tyrosine	Trace	Trace	Trace	0.17	1.08	0.67
Phenylalanine	0.42	0.50	0.67	0.42	1.66	1.25
IV	0.00	0.00	0.00	0.00	0.00	0.05
III	0.00	0.00	0.00	0.00	1.75	0.17
Hydroxylysine	Trace	0.20	0.00	0.08	0.42	0.58
Lysine	0.75	0.92	1.00	1.25	2.08	2.08
Histidine	11.66	11.16	9.66	12.33	11.50	15.24
II	3.08	4.08	0.00	0.50	3.75	6.41
Ammonia	14.00	16.58	31.32	13.33	18.41	13.58
I	3.08	2.58	2.16	1.67	1.75	1.75
Arginine	22.66	21.16	44.98	52.40	55.81	81.13
Total	350.46	324.25	301.94	331.04	362.34	388.21

* Harvested in August, October, and December as winter hardiness was developed. The results are expressed as micromoles of amino acid per gram of lyophilized (dry) tissue.

Figure 2 shows a typical eluto-gram from an alfalfa root extract. Table III summarizes the amount of free amino acids determined by column chromatography in non-hardy and hardy alfalfa roots harvested in August, October, and December 1958. Some of the amino acids showed a greater change than others as the cold weather approached. Arginine increased 246 % from August to December in the non-hardy variety but the increase was 363 % in the hardy variety. Alanine showed the same type of increase. In most cases the amount of each amino acid was approximately the same in the hardy and non-hardy variety during the active growing period of August, but during the period of October to December several of the many amino acids increased significantly more in the hardy variety than in the non-hardy.

It is interesting to note that many of the amino acids accumulated in higher concentrations as plant growth was retarded by decreasing temperatures during the fall. This appears to be a factor which aided in the identification of some amino acids not ordinarily detected in plants. Hydroxylysine and hydroxyaspartic acid are two amino acids whose characterizations will be reported elsewhere.

It will be noted in figure 2 that the peaks labeled ASP-NH_2, GLU-NH_2, and SER contain between one-half and three-fourths of the total amino acids present and that they were not resolved from one another. A second analysis of these extracts after mild acid hydrolysis of the amide showed that nearly all of the amide peak was due to asparagine.

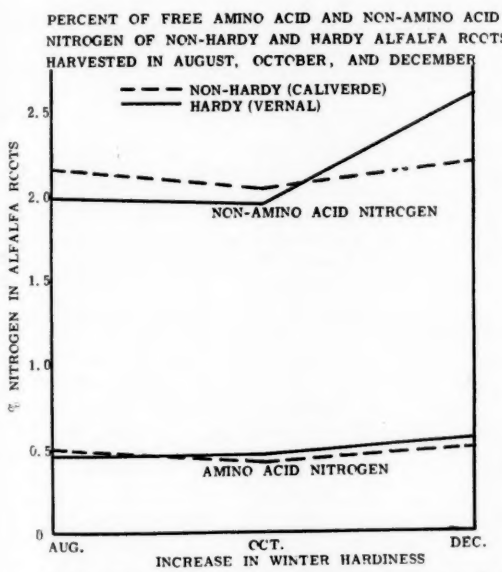

FIG. 3. Per cent of free amino acid and non-amino acid nitrogen in non-hardy (Caliverde) and very hardy (Vernal) alfalfa roots sampled in August, October, and December 1958. The per cent values are given on a dry weight basis. The root nitrogen (obtained by the Kjeldahl method) of these alfalfa varieties was the sum of the amino acid and non-amino acid nitrogen fractions. The amino acid nitrogen values were obtained from quantitative data received from the amino acid analyzer.

The total nitrogen, free amino acid nitrogen, and non-amino acid nitrogen content of the non-hardy and hardy alfalfa roots harvested in August, October, and December are presented graphically in figure 3. The nitrogen in both of these fractions was lower in the hardy variety than in the non-hardy variety during active growth in August. As hardiness began to develop in October, the non-amino acid nitrogen remained about constant and the amino acid nitrogen increased slightly in the hardy variety. By the time hardiness had reached a maximum in December (2), the total amino acids had increased by 20 % in the roots of the hardy variety and the non-amino acid nitrogen had increased 31 % as compared to the August period. In contrast, the non-hardy variety changed very little in both these nitrogen fractions between the August and December periods. The total free amino acid nitrogen represented only about a fifth the total nitrogen found in the roots so the 20 % increase in the free amino acids can account for only a fraction of the 31 % increase in the non-amino acid nitrogen found in the hardy roots in December.

These results are in accord with the observation of Siminovitch and Briggs (11) with the live bark of Black Locust tree. They found that the water-soluble proteins increased in concentration in the bark as hardiness was developed in the fall. They also concluded (1) that there was a marked shift in the electrophoretic patterns between the midsummer proteins and winter proteins. These observed changes showed relatively large shifts in the concentration of two or three of the components relative to the other components of the total protein and not in the number of components present. The increase in total nitrogen from the hardy alfalfa roots observed in the present work would suggest an increase in a protein fraction. The specific increase in some of the free amino acids might be a result of a change in protein synthesis as winter hardiness develops. Thus, an increase, as well as a shift in the type of proteins, may be responsible in part for winter hardiness and the protection of the plant cells against the damage from low temperatures. The non-hardy variety probably is not able to make these changes rapidly enough, or to the proper extent, to protect its cells against the damaging effects of severe winters.

SUMMARY

Studies were carried out from 1957 to 1959 on the free amino acids, organic acids, and sugars in Caliverde, Buffalo, and Vernal alfalfa as related to winter hardiness. The alfalfa varieties which varied in winter hardiness, were planted in the early spring and roots were sampled in August, October, and December. The top 4 inches of the roots were washed, frozen in liquid air, finely ground, lyophilized, and

stored in a desiccator for analysis. A semi-quantitative analysis for amino acids, organic acids, and sugars by paper chromatography of acetone-water extracts of lyophilized alfalfa roots did not show significant differences related to hardiness.

Amino acid analysis by column chromatography showed a 20 % increase in free amino acids from August to December in the roots of the most hardy variety. This was associated with a 31 % increase in non-amino acid nitrogen over the same period. The non-hardy alfalfa variety showed little change in both nitrogen fractions as hardiness developed. Column chromatography revealed several unknown components which reacted with ninhydrin. Two of these were identified as the amino acids hydroxyaspartic and hydroxylysine.

Literature Cited

1. Briggs, D. R. and D. Siminovitch. 1949. The chemistry of the living bark of the Black Locust tree in relation to frost hardiness. II. Seasonal variations in the electrophoresis patterns of the water soluble proteins of the bark. Arch. Biochem. Biophys. 23: 18–28.
2. Bula, R. J., D. Smith, and H. J. Hodgson. 1956. Cold resistance in alfalfa at two diverse latitudes. Agron. Jour. 48: 153–156.
3. Jansen, G. 1929. The relation of organic root reserves and other factors to the permanency of alfalfa stands. Jour. Amer. Soc. Agron. 21: 895–911.
4. Levitt, J. and G. W. Scarth. 1936. Frost-hardening studies with living cells. Can. Jour. Research C14: 267–305.
5. Levitt, J. and D. Siminovitch. 1940. The relation between frost resistance and the physical state of protoplasm. I. Can. Jour. Research C18: 550–561.
6. McGee, C. R. 1935. A search for factors determining winter hardiness in alfalfa. Jour. Amer. Soc. Agron. 27: 685–698.
7. Moore, S., D. H. Spackman, and W. A. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins—an improved system. Anal. Chem. 30: 1185–1190.
8. Rosa, J. R. 1921. Investigations on the hardening process in vegetable plants. Missouri Agr. Exp. Sta. Res. Bull. 48: 3–97.
9. Scarth, G. W. 1941. Dehydration injury and resistance. Plant Physiol. 16: 171–179.
10. Scarth, G. W. and J. Levitt. 1937. The frost-hardening mechanism of plant cells. Plant Physiol. 12: 51–78.
11. Siminovitch, D. and D. R. Briggs. 1949. The Chemistry of the living bark of the Black Locust tree in relation to frost hardiness. I. Seasonal variations in protein content. Arch. Biochem. Biophys. 23: 8–17.
12. Siminovitch, D. and D. R. Briggs. 1953. Studies on the chemistry of the living bark of the Black Locust in relation to its frost hardiness. III. The validity of plasmolysis and desiccation tests for determining the frost hardiness of bark tissue. Plant Physiol. 28: 15–34.
13. Siminovitch, D. and D. R. Briggs. 1953. Studies on the chemistry of the living bark of the Black Locust tree in relation to frost hardiness. IV. Effects of ringing on translocation, protein synthesis, and the development of hardiness. Plant Physiol. 28: 177–200.
14. Siminovitch, D. and J. Levitt. 1941. The relation between frost resistance and the physical state of protoplasm. II. Can. Jour. Research C19: 9–20.
15. Siminovitch, D. and G. W. Scarth. 1938. A study of the mechanism of frost injury to plants. Can. Jour. Research C16: 467–481.
16. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190–1206.
17. Stein, W. H. and S. Moore. 1954. The free amino acids of human plasma. Jour. Biol. Chem. 211: 915–926.
18. Tottingham, W. E. 1931. Tests of Chibnal's method of extraction for investigating winter hardiness in plants. Plant Physiol. 6: 167–176.

FREE AMINO ACIDS IN RED CLOVER AS RELATED TO FLOWERING AND WINTER SURVIVAL [1, 2]

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Red clover is a short-lived perennial legume that lacks the winter hardiness found in legumes like sweet clover, alfalfa, or white clover (3, 7). One of the more difficult problems in the culture of red clover in the northern areas is the weakening or killing of stands during the winter. One of the factors associated with killing during the first winter appears to be flowering. Bird (2) and Smith (9) have shown that plants within a population which do not flower during the seedling year will survive the first winter better than plants that flower. Date of seeding and daylength have a significant influence on the amount of flowering that occurs during the seedling year and on survival over winter (4, 9).

Willard and Lewis (12), Willard (13), Allen and Kuhn (1), and Klebesadel and Smith (5) have shown that clipping medium red clover following harvest of the grain companion crop markedly improved stands and yields the following year. It has been suggested that presence of the grain stubble and weeds might aid in the development of high humidity conditions near the soil surface which would increase the incidence of disease. However, Therrien and Smith (10) have concluded that the important factor probably is that clipping removes the elongating tillers and flowering stems. They found that prevention of flowering by removing flower buds, flowering stems and/or flowering tillers resulted in greater vegetative vigor and improved winter survival. Plants allowed to flower freely in the seedling year in spaced populations had a smaller number of nonflowering crown tillers, a smaller width of crown, a lower dry weight of crown, root and total available carbohydrates in the roots, less root branching, a slightly lower percentage of total available carbohydrates in the roots, and more winter killing during the first winter than plants that were prevented from flowering.

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[2] Published with approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and from the Herman Frasch Foundation.

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MATERIALS AND METHODS

Spaced populations of Wisconsin common medium red clover were established, as described by Smith (9), from seedings made on May 15, 1957, at Madison, Wis. Several management treatments were applied to these seedling plants as described previously by Therrien and Smith (10). Plants from two of these treatments were used for the present biochemical study. The plants in one of the treatments were allowed to grow and flower freely throughout the entire seedling season. In the other treatment, all tillers were cut off with a sharp knife close to the crown as they began to elongate into stems. This treatment was initiated on July 11 and repeated at weekly intervals until August 31. These were treatments 1 and 3, respectively, in the results presented by Therrien and Smith (10). Approximately ten plants sampled at random from one replicate of each of these treatments were removed from the field on November 27, 1957. The soil surface was beginning to freeze at that time. The upper 4 inches of the root from each plant was frozen in liquid air, finely ground, lyophilized, and extracts made for analysis as described previously (11). The prepared extracts were chromatographed on the Beckman/Spinco amino acid analyzer (6, 8).

RESULTS AND DISCUSSION

Table I shows the analysis of the free amino acids in medium red clover roots sampled from the field on November 27, 1957, resulting from two management treatments affecting winter survival. Twenty-three components were detected by this method.

A graphic representation of some of the free amino acids which increased significantly in the roots as a result of removing the elongating tillers from the plant is shown in figure 1. It was observed that aspartic acid, valine, isoleucine, citrulline, and leucine increased more than double, whereas, asparagine increased about 2.5 times the amounts found in the plants allowed to flower freely. Glutamic acid, lysine, and glycine also had significant increases. It should be noted that there was no glutamine in the roots from either treatment. The extraction and analysis of the free amino acids from these legume roots could be duplicated within $\pm$ 5 %.

TABLE I

FREE AMINO ACIDS AS DETERMINED BY COLUMN CHROMATOGRAPHY IN RED CLOVER ROOTS*

AMINO ACIDS	μ/MOLE AMINO ACID/G DRY TISSUE FROM: PLANTS ALLOWED TO FLOWER FREELY	PLANTS WITH ELONGATING TILLERS REMOVED
Elution order		
Hydroxyaspartic	0.58	0.42
Aspartic	8.00	16.58
Threonine	4.91	3.66
Serine	5.50	7.25
Asparagine	121.11	261.23
Glutamine	0.00	0.00
Proline	14.91	16.33
Glutamic	7.00	9.00
Citrulline	0.83	3.58
Glycine	1.16	1.83
Alanine	5.58	4.83
Valine	3.16	7.08
Methionine	0.00	0.00
Isoleucine	2.00	4.25
Leucine	1.50	3.41
Tyrosine	Trace	Trace
Phenylalanine	Trace	0.75
Hydroxylysine	0.33	0.33
Lysine	1.16	1.92
Histidine	14.83	13.33
Ammonia	34.90	36 15
I	0.00	0.33
Arginine	22.07	20.41
Total	249.53	412.67

* Resulting from two management treatments affecting winter survival sampled from the field on Nov. 27, 1957.

There was an overall increase in total free amino acids of 40 to 50 % in the roots of plants from which tillers had been removed throughout the season as compared to the plants allowed to flower freely. This management treatment also was reflected in a significant increase in root and crown development and in winter survival (10). Survival during the first winter for the plants that were allowed to flower was only 42 % as compared to 74 % for the plants that had the tillers removed. Although there was a large increase in free amino acids with removal of the elongating tillers, Kjeldahl analyses revealed only a 4.6 % increase in total nitrogen of the roots. The increase in free amino acids did not change the percentage of total protein in the root. The large increase in certain amino acids, but not in others, may suggest a hydrolysis of some proteins and resynthesis to other specific proteins without changing the percentage of proteins in the roots.

Analyses of these same extracts by paper chromatographic methods did not show as pronounced a difference between the hardy and non-hardy red clover roots as was detected by column chromatography. Many more components were observed by the latter method.

SUMMARY

Plants of Wisconsin medium red clover that had been established in spaced populations in mid-May 1957, were removed from the field in late November for chemical analysis. The plants had either been allowed to grow and flower freely during the seedling year or had been prevented from flowering by removing elongating tillers close to the crown at weekly intervals until late August. Results reported previously (10) showed that prevention of flowering in the seedling year resulted in greater crown and vegetative development and higher winter survival.

Root sections from the plants dug in late November 1957, were analyzed for their content of 23 free amino acids and for total nitrogen. Column chromatography showed a 40 to 50 % increase in amino acids in the hardier plants which were prevented from flowering as compared to the plants allowed to flower but several specific amino acids failed to show this change. This large increase in amino acids was not associated with a change in the total nitrogen of the red clover roots.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. ALLEN, R. J., JR. and A. O. KUHN. 1955. Seedling year management of medium red clover, *Trifolium pratense* L. Maryland Agr. Exp. Sta. Bull. 453.

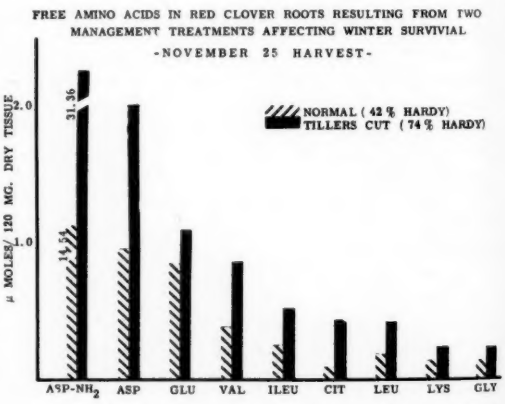

FIG. 1. Nine free amino acids which showed marked quantitative changes in red clover roots resulting from two management treatments affecting winter survival. Plants were allowed to grow and flower freely throughout the summer, or they had the elongating tillers removed weekly from July 11 to August 31. The plants were sampled Nov. 27, 1957.

2. BIRD, J. N. 1948. Early and late types of red clover. Sci. Agr. 28: 444–453.
3. BULA, R. J. and DALE SMITH. 1954. Cold resistance and chemical composition in overwintering alfalfa, red clover, and sweet-clover. Agron. Jour. 46: 397–401.
4. ERMILOV, G. B. 1954. The effect of a short day on the growth and winter hardiness of red clover. Doklady Akad. Nauk. S.S.S.R. 96: 1061–1064. (Herb. Abs. 24: 1210. 1954).
5. KLEBESADEL, L. J. and D. SMITH. 1958. The influence of oat stubble management on the establishment of alfalfa and red clover. Agron. Jour. 50: 680–683.
6. MOORE, S., D. H. SPACKMAN, and W. A. STEIN. 1958. Chromatography of amino acids on sulfonated polystyrene resins—an improved system. Anal. Chem. 30: 1185–1190.
7. RUELKE, O. C. and D. SMITH. 1956. Overwintering trends of cold resistance and carbohydrates in medium red, ladino, and common red clover. Plant Physiol. 31: 364–368.
8. SPACKMAN, D. H., W. H. STEIN, and S. MOORE. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190–1206.
9. SMITH, D. 1957. Flowering response and winter survival in seedling stands of medium red clover. Agron. Jour. 49: 126–129.
10. THERRIEN, H. A., and D. SMITH. 1960. The association of flowering habit with winter survival in red and alsike clover during the seedling year of growth. Can. Jour. Plant Sci. 40: 335–344.
11. WILDING, M. D., M. A. STAHMANN, and D. SMITH. 1960. Free amino acids in alfalfa as related to cold hardiness. Plant Physiol. 35: 726–732.
12. WILLARD, C. J., and R. D. LEWIS. 1947. Effect of combined straw on new seedlings. Paper Trade Jour. 125: 1–4.
13. WILLARD, C. J. 1948. Combined straw injurious to clover seedlings. Paper Trade Jour. 126: 57–59.

DIFFERENTIAL EFFECTS OF 2,3,6-TRICHLOROBENZOIC ACID ON GROWTH AND GEOTROPIC CURVATURE OF AVENA COLEOPTILES[1,2]

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INTRODUCTION

Selective inhibition of geotropic curvature of Avena coleoptiles and other seedlings has been reported in several recent papers. Vander Beek (11) observed that stems of oats, barley, cucumber, and other seedlings failed to assume the normal vertical position when the seedlings were grown in darkness in dilute solutions of 2, 3, 6-trichlorobenzoic acid or 2, 6-dichlorobenzoic acid. Concentrations from 1×10^{-4} to 1×10^{-6} M were effective. The structural requirement for the activity of repression of geotropic curvature appeared to be associated with the two and six positions of the benzene rings. A number of investigators (3, 6, 7, 10) have found that naphthylphthalamic acid caused disproportional inhibition of growth and geotropic curvature particularly of shoots and roots of corn and pea seedlings (10). An explanation of the means of action of this compound has not, as yet, been presented.

Vander Beek (11) grew oat and barley seedlings to the age of 96 hours in solutions of chlorinated benzoic acids and quantified the effects on geotropic inhibition by measuring the angle between the long axis of the seed and a line parallel to the apical half of the coleoptile. The present study is concerned with the effects of 2, 3, 6-trichlorobenzoic acid (TCBA) on elongation and curvature responses of Avena coleoptiles isolated from 72 hour old seedlings.

MATERIALS AND METHODS

Avena sativa L. seeds, Victory Strain (U. S. Dept. of Agriculture, C. I. 2020)[3] were used in these experiments. The seedlings were grown on filter paper strips immersed in distilled water which previously had been aerated. Additional details of the growing method are described elsewhere (14). Only seedlings that were 72 hours old and that had 30 mm (± 2 mm) coleoptiles were used.

Growth measurements were made by two methods. In one group of experiments the coleoptiles were iso-

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[2] Supported in part by a grant from the National Institutes of Health.

[3] Supplied by the U. S. Department of Agriculture.

lated from the seeds and primary leaves. The apical 5 mm sections were discarded; the second 5 mm sections were used. These sections were floated in petri dishes containing the media (20 sections/20 ml solution). All sections were randomized in distilled water and then floated on the experimental media. Control sections grew to an average length of 6.5 mm during the growth period. During cutting and transferring the sections were exposed to red neon light (wave lengths longer than 6,074 A), which previously had been observed to have no effect on the elongation of floating sections. The sections were allowed to elongate in the dark for 24 hours. At the end of this period, their lengths were measured with a micrometer in the ocular of a dissecting microscope. Each point on the curves obtained from this type of measurement represents an average of 40 or more sections.

In most of the growth measurements, apical 15 mm coleoptile sections were used. These sections had intact apices, but the leaves were pulled out. Six such sections were placed in each of two racks which held them in the upright position and exposed only the cut basal ends to the medium. (Each point on the curves obtained by this method represents an average of 6 measurements.) Both racks were placed in a transparent chamber to maintain high humidity. The sections were photographed in flashes of red light (wave lengths above 6,000 A) at 30 minute intervals with a time-lapse apparatus. Measurements of lengths were made from the negative. The basal medium contained only aerated distilled water and the indicated concentrations of TCBA.

Curvature measurements also were made on apical 15 mm coleoptile sections. These were isolated and mounted on sections of stainless steel needles with stops 5 mm from one end. The individual coleoptile holders were fitted into lucite block containers (fig 1). Only the cut basal ends of the coleoptiles were exposed to the aerated distilled water or other solutions. The coleoptiles were left for 2 hours in the holders in upright position before they were geotropically stimulated. After geotropic exposure and curvature development time, the angles of curvature were measured from negatives which were made by shadowgraphing. The entire procedures were carried out at a temperature of 22° C (± 1°).

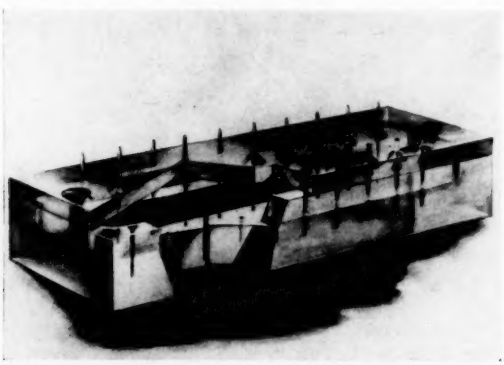


FIG. 1. Diagram of lucite holder which was used to expose the coleoptile bases to various solutions and for geotropic stimulation.

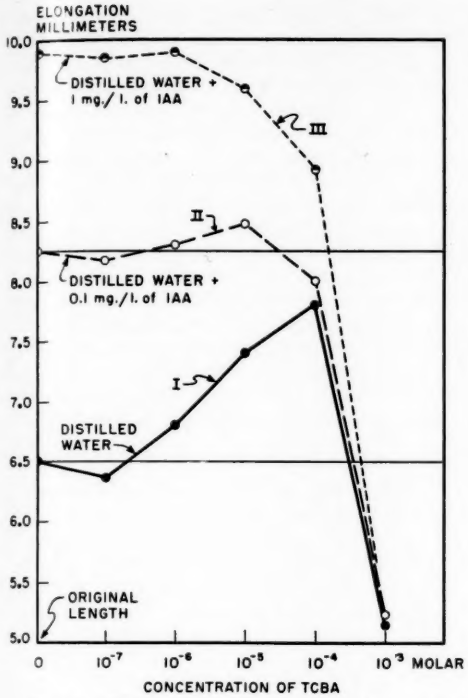
RESULTS

Curve I (fig 2) shows the elongation of 5 mm floating coleoptile sections induced by 1×10^{-7} M to 1×10^{-3} TCBA. These sections were grown in aerated distilled water with the indicated concentrations of TCBA. Sections grown in 0.01 M phosphate buffer at pH 7.0 in the absence of exogenous IAA gave similar results. Control sections increased from 5.0 mm to 6.5 mm during the growth period. It is evident from this curve that concentrations of TCBA in the range 1×10^{-6} M and 1×10^{-4} M stimulated 5 mm coleoptile section elongation with the maximum effect occurring at 1×10^{-4} M. Higher concentrations of TCBA caused complete inhibition of the elongation of floating sections. When IAA was added to medium (either 0.1 or 1.0 mg/l) the TCBA in these concentrations had no stimulatory effect on the elongation of floating sections, but complete inhibition of growth was induced by 1×10^{-3} M TCBA. (See curves II and III in fig 2.)

The curves in figure 3 show the effects of several concentrations of TCBA on the growth of 15 mm coleoptiles with intact apices. Only the cut basal ends of these coleoptiles were exposed to the solutions. One $\times 10^{-6}$ M TCBA produced no effect on the elongation of 15 mm sections. According to the curves in figure 2, this concentration also had no effect on the growth of floating sections. All the other concentrations of TCBA increased growth of 15 mm sections during the first 4 to 6 hours, while concentrations of 1×10^{-4} and 1×10^{-5} M stimulated elongation for the entire 20 hour period. The two highest concentrations, 1×10^{-3} M and 2×10^{-3} M, caused a definite inhibition of growth during the last 14 hours. It is relevant to note that these stimulatory effects of TCBA were observed in the presence of endogenous auxin while similar concentrations of TCBA in the presence of the indicated concentrations of exogenous IAA and no such effect on the growth of floating sections (see fig 2).

The effects of TCBA on geotropic curvature are presented in figure 4. In these experiments, the curvatures were measured at the end of the indicated duration in the horizontal position. The curves labelled water and agar are the controls. A layer of agar 3 mm deep on top of the coleoptile holders caused a slight inhibition of upward bending. (The agar control is related to the results shown in fig 5.) Concentrations of 1×10^{-5} M and 1×10^{-6} M TCBA have little effect on upward bending. The higher of these two concentrations caused a slight inhibition of bending with the longer exposures.

As shown by the results in figure 3, 1×10^{-3} M and 1×10^{-4} M TCBA caused a definite increase in growth during the first 8 or more hours. The data



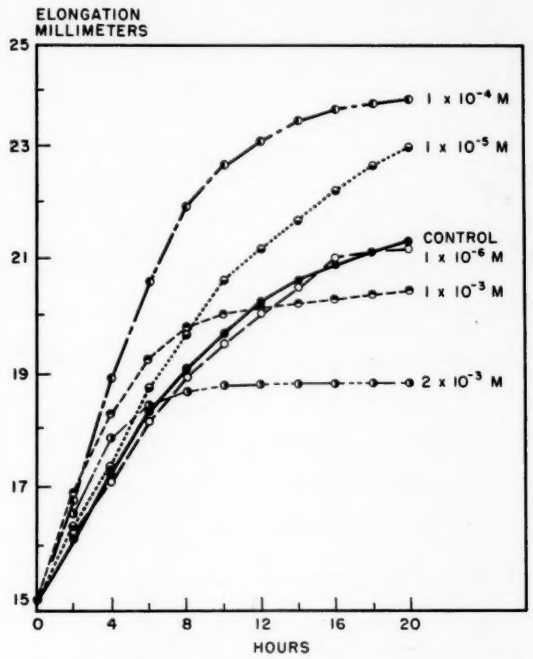


FIG. 2. Effects of various concentrations of 2,3,6-trichlorobenzoic acid on 5 mm floating Avena sections. Each point represents the average length of 40 or more sections. Curve I. Elongation of floating Avena sections in aerated distilled water plus the indicated concentrations of TCBA. Curve II. Elongation of floating Avena sections in 0.1 mg/l of IAA in aerated distilled water plus the indicated concentrations of TCBA. Curve III. Elongation of floating Avena sections in 1.0 mg/l of IAA in aerated distilled water plus the indicated concentrations of TCBA.

FIG. 3. Growth curves of 15 mm Avena coleoptile sections with intact apices showing the effects of various concentrations of TCBA. Each point represents the average of six or more values.

presented in figure 4 show that these same concentrations of TCBA mediated an effective inhibition of negative curvature. Vander Beek (11) found that similar concentrations were effective in his experiments. Actually, the curvature inhibition was more extensive than is apparent from the curves in this figure. When coleoptiles were exposed to 1×10^{-4} M TCBA and left in the holders in the upright position for 2 hours or more, almost all the coleoptiles developed curvatures (see Vander Beek 11). The zone of this randomly directed curvature was in the apical region, and the magnitude of the individual curvatures was extremely variable. The average bending of coleoptiles after 2 hours in the vertical position with the basal ends exposed to 1×10^{-4} M TCBA was from 12 to 15 degrees. Since the coleoptiles were left in the upright position before they were geotropically stimulated, small curvatures invariably developed. If the curves for 1×10^{-3} and 1×10^{-4} TCBA in figure 4 were corrected for this chemically induced bending, the magnitude of the geotropic inhibition would be slightly greater.

Some of Larson's recent work (5) has again directed attention to the fact that maximum geotropic stimulation is obtained when plant structures are exposed at an angle greater than horizontal. Roots pointing upward such that the obtuse angle between a vertical line and the longitudinal axis of the root is 135° (angle of stimulation) manifest the optimum geotropic curvature. The upper curve in figure 5 was obtained from apical coleoptile segments that were stimulated by orienting the apex in a downward direction such that the obtuse angle between a vertical line and the longitudinal axis of the coleoptile was 135°. Preliminary results had demonstrated that stimulation angles of 120° to 135° gave the maximum negative bending in a given length of time. (Compare the upper curve in fig 5 with the control curve labelled agar in fig 4.) For 90 minutes of exposure, a stimulation angle of 120° was slightly more effective in the induction of curvature than a stimulation angle of 135°. For angles of stimulation under 90°, the magnitude of geotropic curvature that developed diminished as the angle of stimulation was decreased.

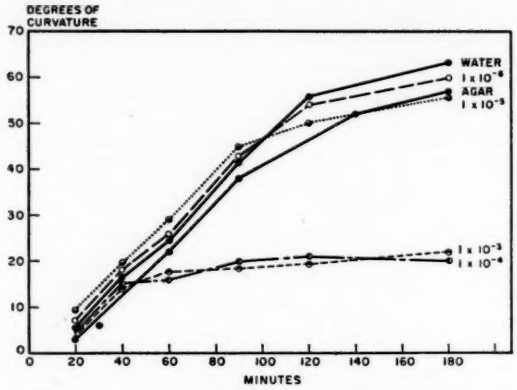
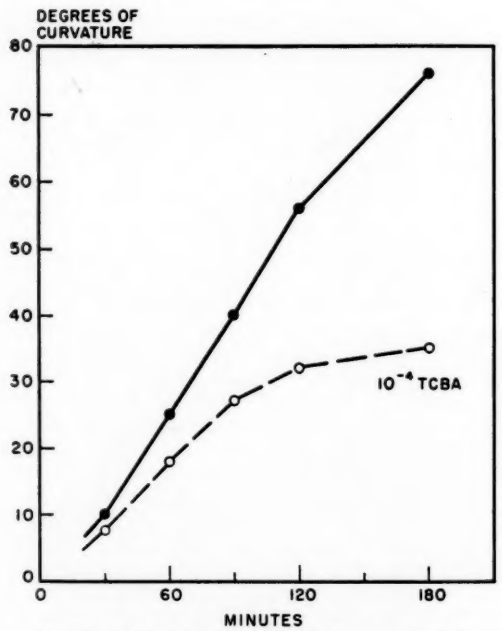


FIG. 4. The effect of TCBA on geotropic curvature of 15 mm apical Avena sections measured at the end of the indicated durations in the horizontal positions. Each point represents the average of 40 or more values. The curve labelled agar is a second control for which the coleoptile holders were covered with a 3 mm layer of agar. The curves showing the effects of 1×10^{-3} and 1×10^{-4} M are extrapolated to the 20 minute reading.

The lower curve in figure 5 shows the geotropic curvature obtained in the presence of 1×10^{-4} M TCBA when the angle of stimulation was 135°. This shows that TCBA effectively inhibits negative curvature of Avena coleoptiles at the indicated angle of stimulation. The curvature developed in this instance is slightly greater than the bending induced by horizontal exposure (angle of stimulation of 90°) in the presence of 1×10^{-4} M TCBA. (See fig 4.)

Two years ago, Brauner and Hager (2) reported results of a study on the effects of low temperatures on the negative geotropic curvature of seedlings of *Helianthus annuus L.* These investigators found that the process of geotropic perception proceeds more efficiently at 4° C than the subsequent development of growth curvatures. This, plus other facts, led Brauner and Hager (2) to the conclusion that the mechanism of geotropic perception is metabolically mediated.

The data shown in figure 6 reveal that 1×10^{-4} M TCBA had no effect on the elongation of 15 mm apical Avena coleoptile sections at 4° C. Comparison of the control curve at 4° C in figure 6 with the control curve at 22° C in figure 3, indicates that coleoptile elongation was extensively inhibited by the low temperature treatment. Similar results



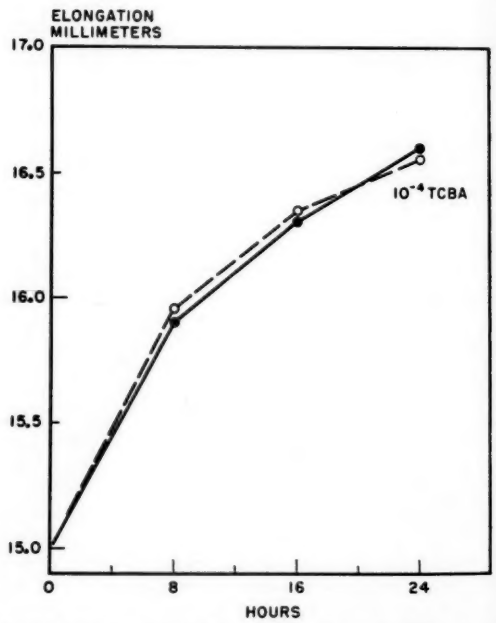


FIG. 5. Geotropic curvature of 15 mm apical Avena sections when stimulated by pointing the coleoptiles in a downward direction such that the obtuse angle between a vertical line and the longitudinal axis of the coleoptiles was 135°. The upper curve represents bending in the absence of TCBA. The lower curve indicates curvature in the presence of 1×10^{-4} M TCBA.

FIG. 6. Elongation of 15 mm apical segments of Avena coleoptiles at 4° C in the presence and absence of 1×10^{-4} M TCBA.

were obtained by Brauner and Hager (2) with Helianthus seedlings.

In contrast to the effect of TCBA on elongation at 4° C, 1×10^{-4} M TCBA definitely inhibited geotropic bending at the same temperature. This observation is verified by the curves in figure 7. Only very small curvatures developed during the first 2 hours in the horizontal position at 4° C. As shown by the curve in figure 7, after 5 hours in the horizontal position the upward curvature of Avena coleoptiles was perceptibly inhibited by 1×10^{-4} M TCBA. With TCBA, 11 hours were required to reach the same curvature attained in 7 hours without TCBA. Thus, the initial rate of bending in the presence of TCBA was only a little more than half the control rate at 4° C. A duration of 24 hours was required to produce the magnitude of curvature normally observed in 3 hours at 22° C. (See control curve in fig 4.)

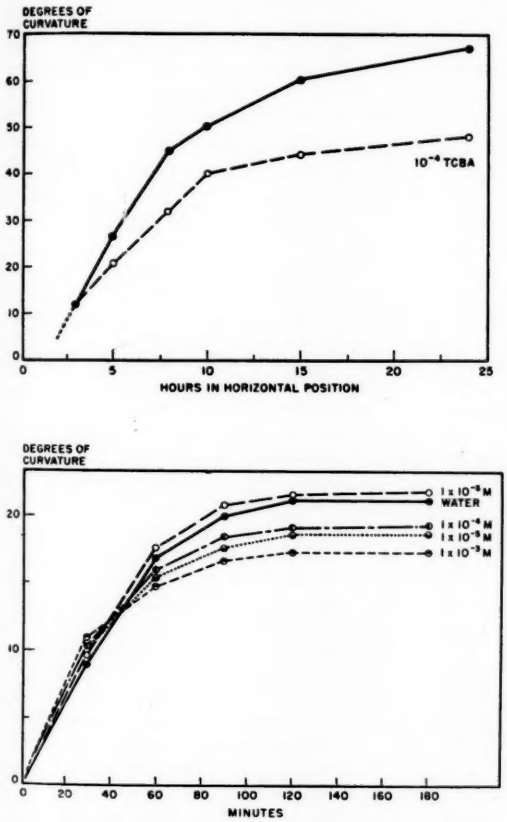


FIG. 7. Geotropic curvature of 15 mm apical segments of Avena coleoptiles at 4° C in the presence and absence of 1×10^{-4} M TCBA.

FIG. 8. Positive phototropic curvatures of 15 mm apical sections of Avena coleoptiles in response to 270 meter-candle-seconds of unilateral white light from an incandescent source. These curvatures were obtained in water and in the presence of the indicated concentration of TCBA.

Since the studies of Vander Beek (11), using TCBA, as well as the work of others with *n*-1-naphthylphthalamic acid (10) did not incorporate phototropic responses, the present study was extended to include positive phototropic bending. The curves in figure 8 show that concentrations from 1×10^{-6} to 1×10^{-3} M of TCBA had no effect on 15 mm coleoptile sections. The curvatures recorded in this figure developed in response to 270 meter-candle-seconds of unilateral white light from an incandescent lamp. When 180 minutes are allowed for bending to develop, all of the curvatures shown in figure 8 fall within ±2.5 degrees of the average. Differences of this magnitude are not statistically significant, partially because TCBA induced greater variation in individual curvatures.

DISCUSSION

The auxin-like activity of 2, 3, 6-trichlorobenzoic acid (TCBA) has been firmly established by several investigators (1, 8, 10, 11, 12, 13, 15), but its use as an inhibitor of geotropic curvature of plant stems has been restricted. Jones, Metcalfe, and Sexton (4) observed that TCBA suppressed the typical geotropic and phototropic responses in rye grass. Vander Beek (11) found that TCBA interfered with the upright growth of shoots of cucumber, barley, oats, and other seedlings. This investigator expressed his results in terms of repression of geotropic curvature and used Avena seedlings for most of his detailed studies. Repression at the age of 96 hours was expressed as the decrease in angle between a line drawn through the long axis of the oat seed and a second line drawn parallel to the apical half of the coleoptile. Vander Beek's photographs show the TCBA treated coleoptiles to be essentially straight but leaning toward the attached seed at some angle between vertical and horizontal. The seedlings were exposed to the test solution during the last 72 hours of growth.

The results reported in this paper are expressed as angles of actual curvature of apical portions of the stimulated coleoptile sections. Exposure to the TCBA was limited to 2 hours of pretreatment in the vertical position plus the duration of the stimulation time. As shown by figure 4, 1×10^{-4} M TCBA was responsible for 60 % inhibition of upward curvature of coleoptiles kept in the horizontal position for 3 hours. This observation explains the fact that Vander Beek's coleoptiles which were treated with the higher concentrations of TCBA were practically straight at the age of 96 hours. There is, however, a slight discrepancy in the concentrations. The curves in figure 4 indicate that TCBA concentrations of 1×10^{-6} to 1×10^{-5} M had no effect on the upward bending in the apical portion of the coleoptile. According to this, Vander Beek's coleoptiles should show a definite upward curvature at the apical end. The long duration of exposure to TCBA used by the pre-

vious investigator could account for this difference in concentration effects. This difference in effectiveness of a given concentration might also be indicative of the fact that two different mechanisms are involved.

Results shown in figures 3 and 4 establish the fact that TCBA at concentrations as high as 1×10^{-3} M increases Avena coleoptile elongation for 6 to 8 hours after its application. A concentration of 1×10^{-4} M accelerates elongation of 15 mm apical coleoptile sections for at least 16 hours and in the absence of exogenous auxin stimulates the growth of floating sections. These same concentrations almost completely inhibit negative geotropic curvature. The explanation of this differential effect is of basic concern.

Geotropic curvature of coleoptiles is generally assumed to be composed of three processes. A: The perception of the force of gravity resulting in B: A unilateral distribution of some substance such as auxin which in turn causes C: Differential rates of elongation and curvature.

TCBA apparently functions as an auxin (1, 8, 9, 10, 11,12). High auxin concentrations become inhibitory in the Avena test presumably because the concentration difference between the opposite sides of the curving structure is decreased. The combination of TCBA and the endogenous auxin in the coleoptiles used in these experiments was quite high. The increased growth rates shown in figure 3 substantiate this proposal. This raises the possibility that the concentration of the combined growth promoting agents is so high that the magnitude of the difference in concentrations on opposite sides is thereby diminished, thus causing a partial masking of the subsequent curvatures. There are two reasons why a masking effect of this nature, if it is involved, has only minor significance. First, phototropic curvature is not extensively inhibited by 1×10^{-3} M TCBA (fig 8). Second, at 4° C, growth is unaffected by 1×10^{-4} M TCBA but geotropic curvature is inhibited (fig 6 and 7). It has been shown by Brauner and Hager (2) that elongation is more sensitive to low temperature than the mechanism of geotropic perception. Thus, TCBA apparently does not inhibit geotropic curvature by influencing the elongation process, because TCBA suppresses curvature but not elongation at 4° C.

Does the TCBA influence geotropic perception or does it alter the unilateral distribution of some growth regulating substance? The results of the low temperature experiments tend to favor the assumption that TCBA inhibits geotropic perception because the rate of lateral transport of auxin very likely is not a limiting factor in experiments lasting 24 hours. Figure 5 shows that an increase in the angle of stimulation from 90° to 135° causes an increase in curvature after 90 minutes. Since there is no direct evidence to indicate or reason to assume that a 135° angle of stimulation suppresses either elongation or lateral transport, it appears likely that TCBA is inhibiting the mechanism of geotropic perception. Stimulation at an angle of 135° induces curvature more effectively than stimulation in the horizontal position. When the angle of geotropic stimulation was 90°, (fig 4) 1×10^{-4} M TCBA inhibited curvature by more than 50 %. With an angle of stimulation of 135°, the same concentration of TCBA mediates a curvature inhibition of only 35 %.

One additional fact is revealed by the data in figures 2 and 3. TCBA in the presence of exogenous IAA (0.1 or 1 mg/l) does not stimulate growth of floating sections, but TCBA does accelerate elongation of sections with intact apices and endogenous auxin. TCBA increases the growth of floating sections in the absence of IAA. (The possibility that TCBA stimulates section elongation in the presence of IAA concentrations less than 0.1 mg/l has not been excluded.) One $\times 10^{-6}$ M TCBA induces a slight increase in the growth of floating sections but has no effect on the elongation of 15 mm apical segments. The explanation of these differences remains for subsequent studies.

Summary

I. The effects of 1×16^{-6} M to 1×10^{-3} M TCBA on the growth of 5 mm floating *Avena sativa* L. sections, on the elongation of 15 mm apical segments, and on the geotropism and phototropism of apical segments were measured.

II. Growth of floating sections in the absence of exogenous IAA, was increased by TCBA concentrations from 1×10^{-6} to 1×10^{-4} M. In the presence of 0.1 mg/l of IAA, these concentrations of TCBA have no effect on floating section elongation. One $\times 10^{-3}$ M TCBA suppressed section elongation in the presence and absence of exogenous IAA.

III. Elongation of 15 mm apical segments was increased by 1×10^{-5} M and 1×10^{-4} M TCBA for a 20 hour period. Concentrations as high as 2×10^{-3} M accelerated elongation for 4 to 6 hours.

IV. Geotropic curvature of 15 mm apical coleoptile segments, stimulated in the horizontal position, was extensively inhibited by 1×10^{-3} M and 1×10^{-4} M TCBA. Lower concentration had no effect on negative geotropism.

V. A stimulation angle of 135° (the obtuse angle between a vertical line and a line of the longitudinal axis of the downward pointing coleoptile) increased geotropic curvature. Geotropic curvature in response to this angle of stimulation was inhibited by 1×10^{-4} M TCBA, but the extent of inhibition is not as large as observed with 90° stimulation.

VI. TCBA (1×10^{-4} M) had no effect on elongation of 15 mm apical segments for a period of 24 hours at 4° C. Geotropic curvature was inhibited by this concentration of TCBA at 4° C.

VII. Positive phototropic curvature was not affected by concentrations of TCBA from 1×10^{-6} to 1×10^{-3} M.

VIII. These results were interpreted to indicate that TCBA in some way inhibits the geotropic perception mechanism.

Literature Cited

1. Bentley, J. A. 1950. Growth regulation effect of certain organic compounds. Nature 165: 449.
2. Brauner, L., and A. Hager. 1958. Versche zur Analyse der Geotropischen Perzeption I. Planta 51: 115–147.
3. Hoffmann, O. L., and A. E. Smith. 1949. A new group of plant growth regulators. Science 109: 588.
4. Jones, R. L., T. P. Metcalfe, and W. A. Sexton. 1954. Relationship between the constitution and the effect of chemical compounds on plant growth. IV. Jour. Sci. Food Agr. 5: 32–38.
5. Larson, P. 1953. Influence of gravity on rate of elongation and on geotropic and autotropic reactions in roots. Physiol. Plantarum 6: 735–774.
6. Mentzer, C., D. Molho, and H. Pacheco. 1950. Relations entra la structure chemique et l'inhibition des tropismes les vegetaux. Bull. soc. chim. biol. 32: 572–582.
7. Mentzer, C. and G. Netien. 1950. Sur un procede permittant de troubler le geotropisme des recines. Bull. mens. soc. linneenne Lyon 19: 102–104.
8. Muir, R. M., C. H. Hansch. and A. H. Gallup. 1949. Growth regulation by organic compounds. Plant Physiol. 24: 359–366.
9. Thimann, K. V. 1952. The role of ortho-substitution in the synthetic auxins. Plant Physiol. 27: 392–404.
10. Tsou Ching, Te-May, R. H. Hamilton, and R. S. Bandurski. 1956. Selective inhibition of the geotropic response by *n*-l-naphthylphthalamic acid. Physiol. Plantarum 9: 546–558.
11. Vander Beek, L. C. 1959. Effect of 2, 3, 6-trichlorobenzoic acid and 2,6-dichlorobenzoic acid on the geotropic and phototropic responses of seedlings of various species. Plant Physiol. 34: 61–65.
12. Veldstra, H. 1952. Researches on plant growth regulators XXI. Rec. trav. chim. 71: 15–32.
13. Wardlaw, C. W. 1953. Action of triiodobenzoic and trichlorobenzoic acids in morphogenesis. New Phytologist 52: 210–217.
14. Wiegand, O. F., and A. R. Schrank. 1959. Regimen for growing Avena coleoptiles. Bot. Gaz. 121: 106–110.
15. Zimmerman, P. W. and A. E. Hitchcock. 1951. Growth-regulating effects of chlorosubstituted derivatives of benzoic acid. Contrib. Boyce Thompson Inst. 16: 209–213.

SEX EXPRESSION AND GROWTH IN MUSKMELON[1, 2]

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Evidence has accumulated from a number of experiments indicating that nitrogen level may modify the sex expression of flowering plants. In general, high levels of nitrogen appear to promote female sex expression, whereas low levels favor male expression. This effect has been most clearly demonstrated in monoecious cucurbits (5, 26) with supporting evidence from studies with hemp, *Cannabis sativa* L. (22, 25) and spinach, *Spinacia oleracea* L. (24).

An increase in the proportion of pistillate flowers of the cucumber, *Cucumis sativa* L., as a result of short-day treatment was observed by Tiedjens (26). Hall (5) was unable to alter flower ratio in the gherkin, *Cucumis anguria* L., with different photoperiods; however, Nitsch, et al (17) later showed that short days promoted pistillate flower development in gherkin and squash, *Cucurbita pepo* L., while long days favored male expression.

Short-day treatment has also been shown to cause a shift toward female sex expression in hemp, *Cannabis sativa* L. (20), Japanese hop, *Humulus japonicus*. Sieb. & Zucc. (21), maize, *Zea mays* L. (23), ragweed, *Ambrosia elatior* L. (8), and cocklebur, *Xanthium Pennsylvanicum* Wallr. (16). These are short-day species with the exception of maize in which flowering is accelerated by short-day treatment (19). Conversely, in spinach, a long-day plant, female sex expression is promoted by long days (24).

A direct effect of auxin on sex expression in the cucumber has been reported by Laibach and Kribben (9). Treatment with naphthaleneacetic acid increased the proportion as well as the total number of pistillate flowers. Heslop-Harrison (6) obtained similar results in hemp as pistillate flowers were formed on genetically male plants in sites normally occupied by staminate flowers. Nitsch et al (17) lowered the node at which the first pistillate flower of *Cucurbita pepo* L. appeared from the 20th to the 9th node with auxin applications. Promotive effects of auxin on flowering also have been reported in both long and short-day species (4, 12, 13, 15). In contrast, inhibitions have been observed in many cases (10, 14).

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[2] Journal paper No. 1428. Purdue University, Agricultural Experiment Station.

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Although sex expression has been studied in several monoecious cucurbits, little information is available on the effect of environment on sex expression in andromonoecious types. In the work reported here a study was made of the effect of nitrogen and auxin under long and short days on the ratio of staminate to perfect flowers in an andromonoecious variety of the muskmelon, *Cucumis melo* L.

Materials and Methods

Nitrogen and Photoperiod Study. Muskmelon seeds of the Queen of Colorado variety were planted September 14, 1957, in plastic pots containing quartz sand thoroughly washed with distilled water. The plants were thinned to one per pot. The nutrient solutions used were basically those of Withrow and Withrow (*27*) modified to contain 20, 100, and 300 ppm of nitrogen with the nitrates of calcium and potassium replaced by the sulfates at the lower nitrogen concentrations. Nutrient solutions were applied every 3rd day for the 1st 2 weeks, then every 2nd day until the last 4 weeks of the experiment when they were applied daily.

Plants were grown under 8- and 16-hour photoperiods with natural daylengths extended by use of incandescent reflector flood lamps giving approximately 200 foot-candles. Daylengths were shortened with black cloth curtains. Photoperiod treatment was started when the 1st leaf was ¾ths fully expanded and continued throughout the experiment. Temperatures were maintained at 65 to 70° F at night and 80 to 85° F during the day.

The nitrogen treatments were arranged in a randomized block design and replicated five times under each photoperiod. Each replication consisted of six pots, two for each nitrogen level.

Staminate and perfect flowers were counted and tagged at anthesis. Flowers were not pollinated and no fruits set during the experiment which was terminated November 28.

Auxin Study. This study was made concurrently with the preceding experiment and methods of plant culture, photoperiods, and temperatures were the same. The nutrient solutions were the same except that only one concentration of nitrogen (300 ppm) was used.

Three concentrations of an auxin, naphthaleneacetic acid (NAA), 0.01, 0.1, and 1.0 ppm were applied in an aqueous solution to the first leaf when about ¾ths fully expanded. One-fourth of the leaf was cut off and the exposed surface immersed in a vial containing 10 ml of the auxin solution. Control plants were treated similarly with distilled water. The leaves remained in the solutions for 7 days.

The pots were arranged in a randomized block design with five replications. Each replication consisted of eight plants, two for each NAA concentration and two controls. Staminate and perfect flowers were recorded at anthesis through November 28.

Table I

Effect of Nitrogen and Photoperiod on Number and Ratio of Staminate to Perfect Flowers in Muskmelon

Nitrogen	Staminate		Perfect		Ratio*	
	L.D.	S.D.	L.D.	S.D.	L.D.	S.D.
20 ppm	49	29	5	2	9.7	15.3
100 ppm	69	42	11	11	6.3	3.8
300 ppm	138	33	37	5	3.7	6.6
LSD 5 %	3	3	1	1	0.8	3.8
1 %	4	5	2	2	1.2	5.5

* Number of staminate to one perfect flower.
L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

Results

Effect on Flower Ratio. The numbers of staminate and perfect flowers increased as the concentration of nitrogen was increased under long days (table I). However, the increase in perfect flowers was proportionately greater resulting in a decrease in the ratio of staminate to perfect flowers. Under short days, the largest number of both flower types and the lowest ratio were obtained at the 100 ppm level of nitrogen. A decline in flower numbers occurred under short days at 300 ppm of nitrogen.

Long days also increased the number of staminate and perfect flowers as well as the proportion of perfect flowers, except at the 100 ppm level of nitrogen (table I). At this concentration, photoperiod had no effect on the number of perfect flowers, while long days increased staminate flowers. Both flower types were produced earlier under short days and at high nitrogen levels. There was little difference in the node at which the first perfect flower appeared.

The number of staminate and perfect flowers was depressed by 0.01 ppm NAA under long days, but the reduction in staminate flowers was proportionately greater and resulted in a decrease in the ratio of staminate to perfect flowers (table II). Higher

Table II

Effect of Auxin and Photoperiod on Number and Ratio of Staminate to Perfect Flowers in Muskmelon

NAA*	Staminate		Perfect		Ratio**	
	L.D.	S.D.	L.D.	S.D.	L.D.	S.D.
Control	129	44	38	5	3.4	8.8
0.01 ppm	79	62	28	11	2.8	5.6
0.1 ppm	67	53	27	6	2.5	8.8
1.0 ppm	61	37	28	4	2.2	9.3
LSD 5 %	5	4	2	2	0.2	2.2
1 %	7	5	3	2	0.3	3.1

* Naphthaleneacetic acid.
** Number of staminate to one perfect flower.
L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

concentrations of NAA had about the same effect on perfect flowers as 0.01 ppm but caused further decreases in staminate flowers.

The results obtained under short days were somewhat different. Treatment with 0.01 ppm NAA promoted flowering and also increased the proportion of perfect flowers. At higher concentrations, there was a decrease in both types and an increase in ratio of staminate to perfect flowers. Concentrations above 0.01 ppm NAA caused a delay in flower development, although there was no difference in the node number at which the first perfect flower developed.

In supplementary work treatment of muskmelons grown under long and short days with 3 % glucose solutions had no effect on flower ratio or plant growth. Applications were made by immersing the cut surface of a leaf in the glucose solution for 7 days. Solutions were applied when the first leaf was fully expanded and again 3 weeks later.

TABLE III

EFFECT OF NITROGEN AND PHOTOPERIOD ON NUMBER OF NODES AND LENGTH OF INTERNODES IN MUSKMELON

NITROGEN	NUMBER		LENGTH, CM	
	L.D.	S.D.	L.D.	S.D.
20 ppm	22	21	6.2	2.9
100 ppm	25	25	8.8	3.8
300 ppm	30	28	9.4	3.6
LSD 5 %	2	2	0.2	0.2
1 %	2	3	0.3	0.3

L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

EFFECT ON PLANT GROWTH. The data in table III show that although higher levels of nitrogen increased the number of nodes about equally under both photoperiods nitrogen had a greater effect on length of internodes under long days. Photoperiod had no effect on number of nodes, but long days more than doubled the length of internodes.

Higher concentrations of nitrogen produced highly significant increases in fresh and dry weight of plants under both daylengths (table IV). Large gains were also obtained in plants grown under long days as compared to short days. Proportionately greater increases in weight were obtained at high nitrogen levels under long days than short days.

TABLE IV

EFFECT OF NITROGEN AND PHOTOPERIOD ON FRESH AND DRY WEIGHT OF MUSKMELON PLANTS

NITROGEN	FRESH WT, G		DRY WT, G	
	L.D.	S.D.	L.D.	S.D.
20 ppm	69	37	6.5	2.5
100 ppm	151	69	14.0	5.2
300 ppm	344	98	33.1	6.5
LSD 5 %	14	5	1.4	0.7
1 %	21	8	2.0	1.0

L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

TABLE V

EFFECT OF AUXIN AND PHOTOPERIOD ON NUMBER OF NODES AND LENGTH OF INTERNODES IN MUSKMELON

NAA*	NUMBER		LENGTH, CM	
	L.D.	S.D.	L.D.	S.D.
Control	30	28	9.6	4.4
0.01 ppm	29	27	8.8	4.2
0.1 ppm	27	30	8.8	4.5
1.0 ppm	25	28	9.6	4.4
LSD 5 %	2	2	0.7	N.S.
1 %	3	N.S.	N.S.	N.S.

* Naphthaleneacetic acid.
L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

Auxin treatment caused a reduction in number of nodes under long days but not under short days (table V). Internode length was not affected by NAA. Fresh weight and dry weight of plants were decreased by auxin under long days (table VI), while weight of plants under short days was not greatly affected.

TABLE VI

EFFECT OF AUXIN AND PHOTOPERIOD ON FRESH AND DRY WEIGHT OF MUSKMELON PLANTS

NAA*	FRESH WT, G		DRY WT, G	
	L.D.	S.D.	L.D.	S.D.
Control	347	99	33.2	6.3
0.01 ppm	224	91	24.1	7.8
0.1 ppm	212	99	22.6	7.6
1.0 ppm	198	84	19.8	6.2
LSD 5 %	30	6	2.6	0.5
1 %	29	8	3.7	0.8

* Napthaleneacetic acid.
L.D. = long day; S.D. = short day.
All data expressed as average of ten plants.

DISCUSSION

In this study, high levels of nitrogen caused an increase in the total number of perfect flowers but the results were somewhat different in a field experiment, conducted by the senior author (Brantley, 2). He reported that moderate amounts of nitrogen caused an increase in the total number of perfect flowers whereas high levels caused a decrease. It is quite possible that the highest level of nitrogen used in the present study was not sufficient to be equivalent to the highest amount used in the field experiment reported by Brantley (2). Considering the effect of nitrogen on flower ratio, the results obtained here agree with

those of Hall (5) and Tiedjens (26) who found that high levels increased the proportion of pistillate flowers in the cucumber.

In contrast to the results reported by other workers on squash (17) and cucumber (26), in the muskmelon, long days rather than short days promoted female sex expression. Thus in short day plants, and some day neutral plants whose development is accelerated by short days, short-day treatment promotes female and depresses male sex expression. In spinach (a long-day plant) and muskmelon (in which flowering is accelerated by long days) long-day treatment promotes female sex expression.

Although the proportion of perfect flowers was increased by auxin under both daylengths, the increases were the result of different responses. Under long days, both flower types were decreased but staminate flowers were depressed to a greater extent. Under short days at 0.01 ppm NAA, both types were increased, with the increase in perfect flowers proportionately greater. In both cases there was a higher percentage of perfect flowers. These results support the evidence presented by Laibach and Kribben (9) and Nitsch et al (17) in monoecious cucurbits.

Heslop-Harrison (7) has suggested that short days, low temperature, and high nitrogen levels may promote female sex expression by increasing auxin concentration. High nitrogen levels have been shown to increase auxin in kohlrabi plants (Avery and Pottorf, 1). Nitsch et al (17) stated that these factors may render plant tissues more sensitive to auxin, an effect that might be obtained through the alleviation of an inhibition.

Evidence has been presented to show that auxin levels are higher under long photoperiods (Leopold, 11; Cooke, 3). It is possible that muskmelons grown under long days have an optimum indigenous level of auxin for flower development and that applied auxin results in a supraoptimal level and a depression of flowering, particularly staminate flowers. An inhibition of microsporogenesis by 2, 4-dichlorophenoxyacetic acid in tomato and watermelon has been reported by Rehm (18). Male sterility lasting a week or more after treatment was obtained, thus altering the functional balance of sex. Muskmelons grown under short days may have a sub-optimal level and respond to low auxin concentrations by an increase in flowering and in the proportion of perfect flowers.

There was no relation between number of nodes developed and flowering response. Growth increases (as indicated by weight of plants) caused by nitrogen and by long-day treatment were accompanied by a shift toward female sex expression. Vegetative growth as influenced by auxin treatment appeared to have no consistent relation to the auxin effect on sex expression.

Summary

Medium and high nitrogen levels and long days each increased the number of staminate and perfect flowers as well as the proportion of perfect flowers in the muskmelon. However, at a concentration of 100 ppm of nitrogen, long days increased staminate flowers while there was no effect of photoperiod on perfect flowers at this nitrogen level.

Photoperiod influenced the response to naphthaleneacetic acid (NAA). The number of staminate and perfect flowers was decreased by NAA under long days with a greater reduction in staminate flowers. Under short days, 0.01 ppm NAA increased both but caused a greater increase in the number of perfect flowers. Higher concentrations under short days caused a depression in total flowering.

Medium and high nitrogen levels and long days increased the fresh and dry weights of plants. The number of nodes was increased by nitrogen but photoperiod had no effect. Length of internodes was increased by long days and by raising the nitrogen level. All concentrations of NAA reduced the number of nodes and weight under long days but had little effect on plants grown under short days.

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Literature Cited

1. Avery, G. S. and L. Pottorf. 1945. Auxin and nitrogen relations in green plants. Amer. Jour. Bot. 32: 666–669.
2. Brantley, B. B. 1958. Effect of nutrition and other factors on flowering, fruiting, and quality of watermelons and muskmelons. Ph.D. Thesis, Purdue University, Lafayette, Indiana.
3. Cooke, A. R. 1954. Changes in free auxin content during the photoinduction of short-day plants. Plant Physiol. 29: 440–444.
4. DeZeeuw, D. and A. C. Leopold. 1956. The promotion of floral initiation by auxin. Amer. Jour. Bot. 43: 47–50.
5. Hall, W. C. 1949. Effects of photoperiod and nitrogen supply in growth and reproduction in the gherkin. Plant Physiol. 24: 753–769.
6. Heslop-Harrison, J. 1956. Auxin and sexuality in *Cannabis sativa*. Physiol. Plantarum 9: 588–599.
7. Heslop-Harrison, J. 1957. The experimental modification of sex expression in flowering plants. Biol. Rev. 32: 38–90.
8. Jones, K. L. 1947. Studies in Ambrosia. IV. Effects of short photoperiod and temperature on sex expression. Amer. Jour. Bot. 34: 371–377.
9. Laibach, F. and F. J. Kribben. 1950. Der einfluss von wuchsstoff auf das geschlecht der bluten bei einer monozischen pflange. Beitr. Biol. Pflanz. 28: 64–67.
10. Lang, A. 1952. Physiology of flowering. Ann. Rev. Plant Physiol. 3: 265–306.
11. Leopold, A. C. 1949. The control of tillering in grasses by auxin. Amer. Jour. Bot. 36: 437–440.
12. Leopold, A. C. and F. S. Guernsey, 1953. Interaction of auxin and temperatures in floral initiation. Science 118: 215–217.

13. LEOPOLD, A. C. and K. V. THIMANN. 1949. The effect of auxin on flower initiation. Amer. Jour. Bot. 36: 342–347.
14. LIVERMAN, J. L. 1955. Physiology of flowering. Ann. Rev. Plant Physiol. 6: 177–210.
15. LIVERMAN, J. L. and A. LANG. 1952. Flowering of long-day plants as a result of auxin treatment (Abstract) A.I.B.S., Madison, Wisconsin.
16. NEIDLE, E. K. 1938. Nitrogen nutrition in relation to photoperiodism in *Xanthium pennsylvanicum*. Bot. Gaz. 100: 607–618.
17. NITSCH, J. P., E. B. KURTZ, J. L. LIVERMAN, and F. W. WENT. 1952. The development of sex expression in cucurbit flowers. Amer. Jour. Bot. 39: 32–43.
18. REHM, S. 1952. Male sterile plants in chemical treatment. Nature 170: 38–39.
19. ROBERTS, R. H. and B. E. STRUCKMEYER. 1938. The effects of temperature and other environmental factors upon the photoperiodic responses of some of the higher plants. Jour. Agr. Res. 55: 633–677.
20. SCHAFFNER, J. H. 1923. The influence of relative length of daylight on the reversal of sex in hemp. Ecology 4: 323–334.
21. SCHAFFNER, J. H. 1923. Sex reversal in the Japanese hop. Bull. Torrey Bot. Club 50: 73–79.
22. SCHAFFNER, J. H. 1925. Experiments with various plants to produce change in sex of the individual. Bull. Torrey Bot. Club 52: 35–47.
23. SCHAFFNER, J. H. 1927. Control of sex reversal in the tassel of Indian corn. Bot. Gaz. 84: 440–449.
24. THOMPSON, A. E. 1955. Methods of producing first-generation hybrid seed in spinach. Cornell Agr. Exp. Sta. Mem. 336: 1–48.
25. TIBEAU, M. E. 1936. Time factor in utilization of minerals by hemp. Plant Physiol. 11: 731–747.
26. TIEDJENS, V. A. 1928. Sex ratios in cucumber flowers as affected by different conditions of soil and light. Jour. Agr. Res. 36: 721–746.
27. WITHROW, R. B. and A. P. WITHROW. 1938. Nutrient solution methods of greenhouse crop production. Ind. Agr. Exp. Sta. Cir. 232: 1–20.

ROLE OF GLYOXYLATE IN BIOSYNTHESIS OF ACIDS IN TOMATO FRUIT[1, 2]

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Previous work by Wang et al (3, 19) on the metabolism and biosynthesis of acids in tomato fruits indicated that malate is synthesized in part by a CO_2 fixation of the $C_3 + C_1$ type. The suggestion was also made that a $C_2 + C_2$ condensation presumably of the Thunberg type may have played an important role in the biosynthesis of fruit acids. The formation of malate in apples by way of CO_2 fixation has also been reported by Allentoff (1). More recently the net synthesis of malate from glyoxylate and acetate through the action of malate synthetase has been demonstrated by Wong and Ajl (21). Glyoxylate is presumably derived indirectly from acetate via the isocitritase reaction (5, 17). The combined operation of these processes, constituting the glyoxylate cycle (11), in castor bean seedlings has been recently reported by Kornberg and Beevers (10).

In light of this development, the biosynthetic mechanism for malate in tomato fruit has been examined by a series of incorporation experiments employing glyoxylate-1- and -2-C^{14} as test substrates. The findings suggested the occurrence of the malate synthetase reaction in tomato fruit and the importance of this reaction in the overall acid metabolism has been evaluated.

MATERIALS AND METHODS

FRUITS: Green mature tomato fruits used in the present work were of the Michigan State forcing variety and were selected on the basis of uniformity in size and degree of maturity. The fruits were picked immediately prior to the experiments.

CARBON 14 LABELED SUBSTRATES: Glyoxylate-1- and -2-C^{14} were synthesized from fumarate-1, 4 and -2, 3-C^{14}, respectively, by a modified version of the methods of Milas and Terry (12) and Radin (16). The radiochemical purity of these compounds was determined by paper chromatography and radioautography.

ADMINISTRATION OF LABELED SUBSTRATES: The labeled substrates were introduced into the fruit as aqueous solutions by means of the modified vacuum infiltration method described by Barbour et al (2). Substrate levels used in both the radiorespirometric and incorporation experiments are summarized in table I.

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[2] This research was supported by a contract from the Atomic Energy Commission (No. AT(45–1)573), published with the approval of the Monograph Publication Committee, Research Paper No. 369, School of Science, Oregon State College.
[3] A portion of the work described herein is taken from the thesis presented by W. P. Doyle for the degree of Doctor of Philosophy at Oregon State College, 1960. Present address: Texaco Research Laboratories, Beacons, New York.
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RADIORESPIROMETRIC EXPERIMENTS: The radiorespirometric technique described by Barbour et al (2) was used in this work to study the utilization of specifically labeled glyoxylate by tomato fruit for the respiratory functions.

INCORPORATION EXPERIMENTS: After administration of the labeled glyoxylate, the metabolism of the glyoxylate by the fruit was followed by the radiorespirometric technique and the incorporation experiment was terminated at the time that the rate of evolution of $C^{14}O_2$ from fruit metabolizing glyoxylate-1-C^{14} had passed its second peak value.

The tomatoes were homogenized and extracted exhaustively with 80 % ethanol. After concentration of the ethanol extract, it was dried over P_2O_5 in vacuo and defatted with petroleum ether. The defatted fruit extract was dissolved in water and passed through a Dowex-50 (H form) column to remove the amino acids from the mixture. Since glyoxylate is unstable in the presence of a basic solution or weakly basic exchange resins, the remaining mixture was acidified and extracted continuously with alcohol-free ether for 64 hours. The ether extract was concentrated and the residue dried over P_2O_5 in vacuo. The mixed acids in the residue were separated on a silica gel column according to the method of Bulen et al (4). The carbohydrates in the aqueous phase of the ether extraction were subjected to radioactivity assay by a persulfate combustion and examined by chromatography and radioautography.

Malic acid was isolated from the eluent of the silica gel column through the formation of its lead salt and the subsequent removal of lead ion with H_2S. The isolated malic acid was then diluted with carrier to a level given in table III and purified by recrystallization from a mixture of dry benzene and acetone. Citric acid was isolated as its calcium salt, and diluted with a prescribed amount of carrier compound (table III).

The amino acids were eluted from the Dowex-50 column with 4 % HCl. After removal of HCl by repeated evaporation of the aqueous solution, the radioactivity of the amino acids was assayed by a persulfate combustion (6). Individual amino acids were separated and their radioactivity estimated by means of paper chromatography and radioautography.

The specific activity of the isolated malic acid was determined by the persulfate combustion method. The isotopic distribution pattern of malic acid was determined in the following manner: A. Malic acid was degraded by a $KMnO_4$ oxidation giving rise to CO_2 corresponding to C-1 and C-4 and acetaldehyde corresponding to C-2, 3 (8). The acetaldehyde was further degraded by the iodoform reaction (9) converting C-3 of malate to iodoform and C-2 to formate. The excess iodine in the reaction mixture was removed by thiosulfate titration and the insoluble iodoform recovered by centrifugation. The formic acid was recovered by steam distillation and was subsequently oxidized to CO_2 with HgO. B. Malic acid was heated at 50° C in 100 % H_2SO_4 thus giving rise to CO from C-1. The CO was converted to CO_2 by passing it over CuO at 400° C (15). The specific activity of C-4 of malate was determined directly by raising the reaction temperature from 50° to 100° C after all the C-1 had been recovered as CO.

The specific activity of the diluted calcium citrate was determined by a persulfate oxidation. The labeling pattern in citrate was determined as follows: A. C-1, 5 were converted to CO_2 and C-6 to CO by the action of 100 % H_2SO_4 (20). The latter was oxidized to CO_2 as described in the malate degradation. The activity in C-2, 3, 4 was obtained by a Van Slyke oxidation (18) of the acetone remaining in the reaction vessel after decarboxylation. B. Citrate was decarboxylated and the resulting acetone trapped as its Deniges complex (20). The specific activity of C-2, 4 was obtained by means of a hypoiodite oxidation (9) of the acetone liberated from this complex. The activity of C-3 was obtained by way of the hydrazoic acid degradation of acetic acid (14) obtained in the iodoform reaction.

RESULTS AND DISCUSSION

In order to evaluate the roles played by the malate synthetase reaction and isocitritase reaction in tomato fruit it is desirable to first examine the participation of glyoxylate in the respiratory function, consequently glyoxylate-1- and -2-C^{14} were employed as test substrates in a set of radio-respirometric experiments. The interval and cumulative radio-chemical re-

TABLE I

RADIORESPIROMETRIC AND INCORPORATION STUDIES ON TOMATO FRUITS CATABOLIZING SPECIFICALLY C^{14}-LABELED SODIUM GLYOXYLATE

SUBSTRATE	RADIOCHEMICAL LEVEL, μC	CHEMICAL LEVEL, MG	AVG TOMATO WT, G	AVG % C^{14} RECOVERY IN CO_2 AT X HRS
		Radiorespirometric studies		
Glyoxylate-1-C^{14}	1.01	19.2	51*	33 (36 hrs)*
Glyoxylate-2-C^{14}	1.12	19.2	51*	7 (36 hrs)*
		Incorporation studies		
Glyoxylate-1-C^{14}	11.2	21	57	32 (34 hrs)
Glyoxylate-2-C^{14}	14.6	26	54	8 (34 hrs)

* Average of three replicate experiments

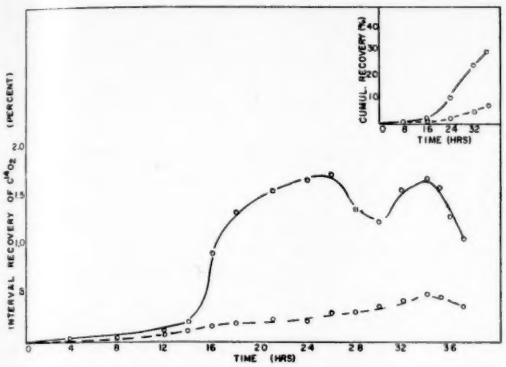


FIG. 1. The interval and cumulative radiochemical recoveries of administered radioactivity in respired CO_2 from tomato fruits metabolizing specifically C^{14} labeled glyoxylate. Glyoxylate-1-C^{14} ————————; Glyoxylate-2-C^{14} — — — — — — — —.

coveries of substrate activity in respired CO_2 derived from these substrates are shown in figure 1. Data given are the average values observed in triplicate experiments. The lag observed in the rate of interval radiochemical recovery may reflect one of the following situations: A. A hindered cellular permeability of substrate, B. a rate-determining set in converting glyoxylate to an activated form, and C. the formation of adaptive enzyme for glyoxylate utilization. It was noted that the interval $C^{14}O_2$ recovery curve for the metabolism of glyoxylate did not resemble those obtained earlier for pyruvate or acetate (*7*). The C-1/C-*2* ratio for glyoxylate radioactivity recovered in CO_2, calculated from the radiochemical recoveries observed at the end of the experiment, was 4.7/1. The C-1/C-*2* ratio observed for acetate was 3.7/1. The marked differences observed in the C-1/C-*2* ratios and in the general profile of the interval radiochemical recovery curves for acetate and glyoxylate suggest strongly that basically different routes are involved in the entry of the respective acids into the respiratory mechanism. One of the most interesting differences in catabolic behavior between these two substrates is that the radiochemical recovery curve for C-1 of glyoxylate possesses two maxima observed in three replicate experiments. Such a behavior may indicate that there exist two catabolic routes available to glyoxylate, one of which is either relying on the product of the first reaction or operating independently but at a much slower rate, resulting in a second peak rate of $C^{14}O_2$ production from glyoxylate carboxyl carbon. Further discussion of these possibilities will be presented in connection with the glyoxylate incorporation data.

The interesting finding in the radiorespirometric studies of glyoxylate utilization prompted the use of C^{14} specifically labeled glyoxylate in incorporation experiments. The distribution of the substrate radioactivity in various fractions of the test fruit is summarized in table II. The relatively large amount of radioactivity found in the amino acid fraction was almost exclusively in the form of glycine and serine. This is not surprising in view of the well known conversion of glyoxylate to glycine (13). It was also noted that the serine isolated from the glyoxylate-*2*-C^{14} experiment was much richer in C^{14} relative to glycine than an analogous comparison in the glyoxylate-1-C^{14} experiment. The latter fact accounts for the observed heavier labeling of the amino acid fraction from glyoxylate-*2*-C^{14} in comparison to that from glyoxylate-1-C^{14}. The preferential incorporation of glyoxylate-*2*-C^{14} radioactivity into serine can

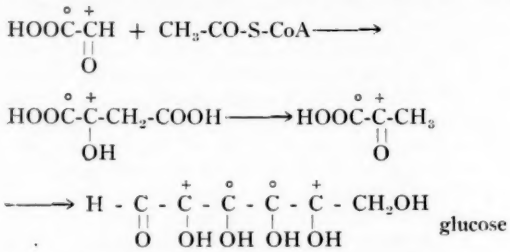
TABLE II

DISTRIBUTION OF RADIOACTIVITY AMONG FRACTIONS ISOLATED FROM TOMATO FRUITS METABOLIZING GLYOXYLATE-1-OR -*2*-C^{14}

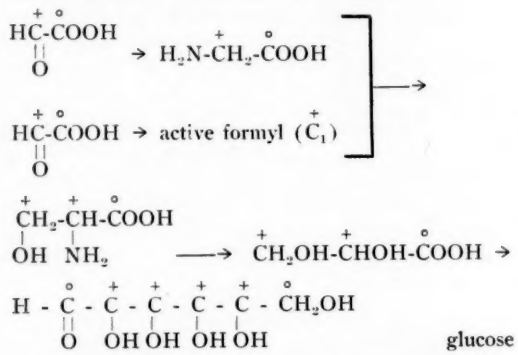
FRACTION	GLYOXYLATE-1-C^{14}		GLYOXYLATE-*2*-C^{14}	
	MICROCURIES	% OF TOTAL	MICROCURIES	% OF TOTAL
Substrate Administered	11.2	100	14.6	100
Pulp	1.11	10	3.17	*22*
Ethanol Extract	5.10	46	8.80	60
Respired CO_2	3.58	32	1.08	8
Fats	0.18	2	0.22	*2*
Acids	2.31	21	3.32	23
Glyoxylate	2.00	18	2.75	17
Acetate	0.00	...	0.00	...
Citrate	0.07	1	0.06	1
Malate	0.06	1	0.06	1
Formate plus Fumarate	Trace	...	0.27	*2*
Unknown	0.10	1	0.2	*2*
Amino Acids	0.68	6	2.38	17
Carbohydrates	1.00	9	2.14	15
Total Recovery	8.86	79	12.32	84

be explained by the role played by C-2 of glyoxylate in the formylation of glycine. Such a series of reactions has been described by Nakada (13).

The carbohydrate fraction in the glyoxylate-1-C^{14} experiment contained 9 % of the total substrate radioactivity in contrast to a value of 15 % observed in the glyoxylate-2-C^{14} experiment. Apparently there exists a mechanism for the preferential incorporation of C-2 of glyoxylate into the carbohydrate skeletons. Bearing this in mind, one can visualize at least two possible routes. One pathway involves the sequence of reactions elucidated by Kornberg et al (10) in which glyoxylate is first condensed with acetyl CoA via the malate synthetase reaction to yield malate. The malate could in turn be decarboxylated to form pyruvate which would give rise to glucose and fructose via reversed glycolysis. Such a sequence is shown:



Partial equilibration of malate with fumarate may introduce additional labeling into C-1 and C-6 of glucose from C-2 of glyoxylate. An alternate mechanism calls for the conversion of serine, being preferentially labeled from glyoxylate-2-C^{14}, to D-glycerate which again by reversed glycolysis could lead to labeled glucose and fructose, as shown below:

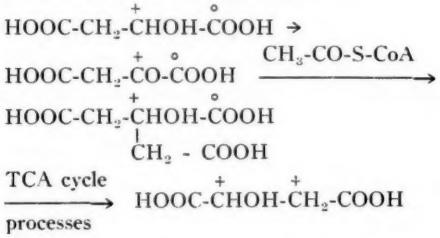


The bulk of the radioactivity in the acid fraction was present in the form of intact glyoxylate. About 1 % of the total substrate radioactivity was located in malic and citric acids isolated in either the glyoxylate-1- or -2-C^{14} experiment. It is also interesting to note that fruit acetate contained no detectable radioactivity in these experiments. In view of the rapid turnover of acetate in tomato fruit (7, 19) and the total duration of the glyoxylate incorporation experiments (34 hrs), it is reasonable to believe that glyoxylate was not converted to acetate to any significant extent. Two per cent of the glyoxylate-2-C^{14} radioactivity was accounted for in the formate-fumarate fraction in contrast to a trace amount of C^{14} detected in the same fraction in the glyoxylate-1-C^{14} experiment.

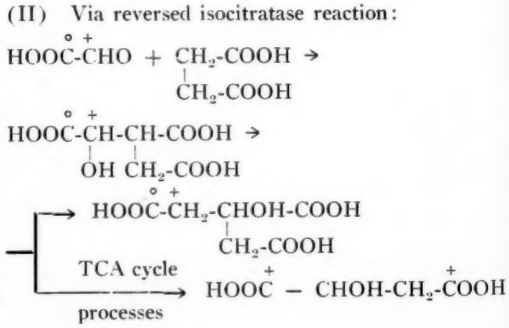
The isotopic distribution patterns observed in malate and citrate are given in table III. In view of the observation that substrate activity was not detected in the fruit acetate and that the only likely mechanism whereby C-1 and C-2 of citrate may become labeled is the condensation of acetyl CoA with oxaloacetate, it was therefore assumed that the observed radioactivity of C-1, 5 and C-2, 4 was in actuality located solely in C-5 and C-4 of citrate, respectively. These patterns represent the observed and that corrected for pool size and dilution. It is evident that the interpretation of the observed labeling patterns could become quite complicated in view of the many possible modes by which a specific carbon in either citrate or malate may become labeled. Nevertheless some qualitative conclusions can be drawn from these results with regard to the metabolic behaviors of glyoxylate in tomato fruit.

In view of the radiorespirometric data observed in the glyoxylate experiment and the important role played by the TCA cycle in tomato respiration (19), it is reasonable to believe that glyoxylate, particularly the aldehyde carbon atom, was incorporated into TCA intermediates prior to oxidation to CO_2. Examination of existing literature revealed that there are at least three possible mechanisms for such an incorporation. The anticipated labeling patterns for citric acid and malic acid are shown:

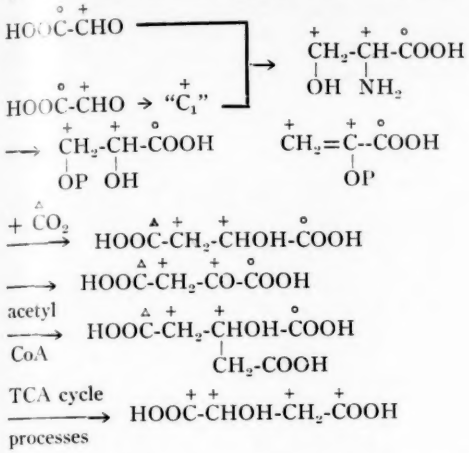
(I) Via malate synthetase reaction:



(II) Via reversed isocitratase reaction:



(III) Via serine and phosphophenol pyruvate:



It is evident that one cannot expect a perfect agreement between the observed labeling patterns with one or more of the foregoing mechanisms in view of the effect of numerous mechanisms for labeling randomization. Some notable mechanisms in this regard are the equilibration of malate with symmetrical C_4 acids and labeling shifting in the TCA cyclic process. It can be recognized, however, that the relatively heavier labelings of C-2 of malate and C-3 of citrate from C-2 of glyoxylate are significant indications for the operation of mechanism (I). The other two mechanisms would have given rise to either labeling exclusively in malate carboxyls (mechanism II) or equal labeling of C-2 and C-3 of malate from C-2 of glyoxylate. The findings in the present work consequently suggest, at least qualitatively, the operation of the malate synthetase reaction in the biosynthesis of C_4 acids in tomato fruit. The significance of the malate synthetase reaction in the overall C_4 biosynthesis, however, cannot be well defined in the present work. The low incorporation of glyoxylate activity into the acid fraction could be the result of either a rapid turnover of glyoxylate carbon atoms in the TCA processes or an insignificant contribution of the malate synthetase reaction in acid metabolism.

The operation of isocitratase reaction in tomato fruit cannot be definitely established in the present study. The detection of C^{14} in C-4 of citrate in the glyoxylate-2-C^{14} experiment may reflect to some ex-

TABLE III

LABELING PATTERNS OF MALATE AND CITRATE FROM TOMATOES METABOLIZING GLYOXYLATE-1- AND -2-C^{14}

SUBSTRATE	NO. C ATOMS	UNCORR. S.A. CPM × 10^{-2} / MM	CORR. S.A.** CPM × 10^{-3} / MM	% DISTRIBUTION
	Malate			
Glyoxylate-1-C^{14}	COOH (C-1)	11.8	51.1	39
	COH (C-2)	0.	0.	0
	CH_2 (C-3)	0.	0.	0
	COOH (C-4)	18.2	78.4	61
	Citrate			
Glyoxylate-1-C^{14}	COOH (C-1)	0. *	0.	0
	CH_2 (C-2)	0. *	0.	0
	COH (C-3)	0.9	0.7	2
	CH_2 (C-4)	0.9	0.7	2
	COOH (C-5)	28.0	21.1	52
	COOH (C-6)	23.6	17.7	44
	Malate			
Glyoxylate-2-C^{14}	COOH (C-1)	4.3	18.1	15
	COH (C-2)	8.9	37.1	32
	CH_2 (C-3)	5.5	23.0	20
	COOH (C-4)	9.1	38.1	32
	Citrate			
Glyoxylate-2-C^{14}	COOH (C-1)	0. *	0.	0
	CH_2 (C-2)	0. *	0.	0
	COH (C-3)	19.9	13.8	40
	CH_2 (C-4)	3.9	2.7	8
	COOH (C-5)	20.8	14.4	42
	COOH (C-6)	5.1	3.6	10

* Assumed to be unlabeled on the ground that substrate activities were not found in fruit acetate.
** Corrected for pool size and dilution in the isolation procedures.

tent the condensation of glyoxylate with succinate in the nature of mechanism II. It is equally true, however, that the labeling could have been the result of randomization of C-3 and C-4 of citrate in the TCA cyclic processes.

Also note that in the present work the C_4 condensing component of citric acid is not symmetrical in nature if one considers the classical biosynthetic mechanism of this acid. This is in contrast to the findings in a previous paper using pyruvate-2-C^{14} as tracing substrate. It is possible that the difference stems from the fact that in the pyruvate-2-C^{14} experiment (3), glyoxylate and acetate were presumably derived from the administered pyruvate and hence resulted in similar specific activities.

The heavy labeling of C-5 of citrate from C-2 of glyoxylate cannot be readily explained by either of the three mechanisms for glyoxylate utilization, although it is possible that reentry of metabolic $C^{14}O_2$ via a fixation mechanism may be one of the factors. Examination of the incorporation data on $C^{14}O_2$ fixation reported in a previous paper led us to believe that the preferential labeling of C-5 of citrate from glyoxylate carbon atoms may involve an heretofore unknown mechanism.

In view of the results from these incorporation experiments further discussion of the radiorespirometric data is warranted. It is possible that two separate sets of catabolic pathways are responsible for the two observed maxima in the radiorespirometric pattern for glyoxylate-1-C^{14} metabolism. First, the decarboxylation of glyoxylate to form either an active formyl group, as evidenced by the preferential incorporation of radioactivity into serine from glyoxylate-2-C^{14} over that found in the glyoxylate-1-C^{14} experiment. This reaction should result in a rapid conversion of C-1 of glyoxylate to CO_2. Second, the indirect oxidation of the glyoxylate carboxyl incorporated previously either into the TCA cycle intermediates and particularly fruit carbohydrates, would be a relatively slower process and hence reflected as a delayed maximum in the interval $C^{14}O_2$ recovery curve.

Summary

Radiorespirometric and incorporation studies of glyoxylate metabolism in tomato fruit have been carried out and reveal the following: A: Glyoxylate can participate in respiratory functions. B: Glycine and serine may be synthesized from glyoxylate presumably via a sequence of reactions similar to those described by Nakada. C: Glyoxylate can be incorporated extensively into the carbohydrates. D: The occurrence of the malate synthetase reaction in tomatoes is indicated by the incorporation of glyoxylate into the malate molecules.

Literature Cited

1. Allentoff, N., W. R. Phillips, and F. B. Johnston. 1954. A C^{14} study of carbon dioxide fixation in the apple. I. The distribution of incorporated C^{14} in the detached MacIntosh apple. Jour. Sci. Food Agr. 5: 231–234.
2. Barbour, R. D., D. R. Buhler, and C. H. Wang. 1958. Identification and estimation of catabolic pathways of glucose in fruits. Plant Physiol. 33: 396–400.
3. Buhler, D. R., E. Hansen, B. E. Christensen, and C. H. Wang. 1956. The conversion of $C^{14}O_2$ and $CH_3C^{14}OCOOH$ to citric and malic acids in tomato fruits. Plant Physiol. 31: 192–195.
4. Bulen, W. A., E. Varner, and R. C. Burell. 1952. Separation of organic acids from plant tissues. Anal. Chem. 24: 187–190.
5. Campbell, J. J. R., R. A. Smith, and B. A. A. Eagles. 1953. Deviation from the conventional tricarboxylic acid cycle in *Pseudomonas aeruginosa*. Biochem. Biophys. Acta 11: 594.
6. Chen, S. L., and K. J. H. Lauer. 1957. Carbon determination in biological material with a persulfate oxidation method. Anal. Chem. 29: 1225–1226.
7. Doyle, W. P., and C. H. Wang. Radiorespirometric studies of glucose catabolism in tomato fruit. Plant Physiol 35: 751–756.
8. Friedmann, T. E. and A. I. Kendal. 1929. The determination of lactic acid. Jour. Biol. Chem. 82: 23–43.
9. Hatcher, W. H. and W. H. Mueller. 1929. The iodimetric estimation of organic compounds. Transactions of the Royal Society of Canada. 23: 35–44.
10. Kornberg, H. L. and H. Beevers. 1957. A mechanism of conversion of fats to carbohydrates in castor beans. Nature 180: 35–36.
11. Kornberg, H. L. and H. A. Krebs. 1957. Synthesis of cell constituents from C_2 units by a modified TCA cycle. Nature 179: 988–991.
12. Milas, N. A. and E. M. Terry. 1925. Oxidation of fumaric and malic acid to tartaric acid. Jour. Amer. Chem. Soc. 47: 1412–1418.
13. Nakada, H. I. and L. P. Sund. 1958. Glyoxylic acid oxidation by rat liver. Jour. Biol. Chem. 233: 8–13.
14. Phares, E. F. 1951. Degradation of labeled propionic and acetic acid. Arch. Biochem. Biophys. 33: 173–185.
15. Racusen, D. W. and S. Aronoff. 1953. Metabolism of soybean leaves: The dark reaction following photosynthesis. Arch. Biochem. Biophys. 42: 25–40.
16. Radin, N. S. 1955. Sodium glyoxylate monohydrate. Biochem. Preparations 4: 60–62.
17. Smith, R. A., I. C. Gunsalus. 1954. Isocitritase: A new tricarboxylic acid cleavage system. Jour. Amer. Chem. Soc. 76: 5002–5003.
18. Van Slyke, D. D. and J. Folch. 1940. Manometric carbon determination. Jour. Biol. Chem. 186: 507–541.
19. Wang, C. H., E. Hansen, and B. E. Christensen. 1953. Conversion of C^{14}-labeled acetate to citric and malic acid in the tomato fruit. Plant Physiol. 28: 741–745.
20. Weinhouse, S., G. Medes, and N. E. Floyd. 1948. Fatty acid metabolism: The conversion of fatty acid intermediates to citrate, studied with the aid of isotopic carbon. Jour. Biol. Chem. 166: 691–703.
21. Wong, D. T. O., and S. J. Ajl. 1957. Conversion of acetate and glyoxylate to malate. Jour. Amer. Chem. Soc. 78: 3230–3231.

RADIORESPIROMETRIC STUDIES OF GLUCOSE CATABOLISM IN TOMATO FRUIT [1, 2]

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The occurrence of various catabolic pathways of glucose in plant materials has attracted considerable interest in recent years (1, 3, 6). In an earlier report (2), studies on the catabolism of glucose in fruit by a radiorespirometric method were described. The radiorespirometric method previously has been successfully applied to other intact biological systems such as microorganisms and insects (11, 13). Essentially, the catabolic pathways for glucose utilization in fruit were detected and estimated by comparing the rates of $C^{14}O_2$ formation from fruit administered with C^{14} specifically labeled glucose samples.

In applying this method to study tomato catabolism, it is important that the labeled substrate is introduced into an intact fruit by a reliable technique so that it will mix rapidly and evenly with a defined amount of endogeneous substrate. Fullfillment of these conditions will then make it possible to compare the rate of $C^{14}O_2$ production from a set of fruit metabolizing concurrently C^{14} specifically labeled substrates. The vacuum infiltration technique described in an earlier paper was found to be satisfactory by examining the reproducibility of a large number of radiorespirometric data in a series of test experiments. In order to understand better the nature of the vacuum infiltration process and to estimate the amount of fruit glucose located at the infiltrated area, isotopic dilution technique has been applied in the present work to further examine the validity of the technique described earlier for substrate administration.

Results of several series of radiorespirometric experiments are also presented to provide experimental evidence in substantiating the assumptions given in the earlier paper (2) for estimating pathway participation in the glucose catabolism of tomato fruit. These assumptions, concerned with the catabolic behavior of acetate, pyruvate, and gluconate in tomatoes, are essential in the derivation of equations for pathway estimation.

MATERIALS AND METHODS

FRUITS: The tomato fruit used in the present work were of the Michigan State forcing variety and test samples, mature but green, were selected on the basis of uniformity in size and degree of maturity. They were picked immediately prior to each of the experiments.

CARBON 14 LABELED SUBSTRATES: Pyruvate-1-, -2-, -3-C^{14} and gluconate-1-C^{14} were obtained from the Nuclear-Chicago Corp. Acetate-1- and -2-C^{14} and glucose-U-C^{14} were supplied by Tracerlab, Inc. Gluconate-2-, -3(4)- and -6-C^{14} were prepared by the method of Moore and Link from the correspondingly labeled glucose samples (8). Glucose-2- and -6-C^{14} were obtained from the National Bureau of Standards through the kind cooperation of Dr. H. S. Isbell. Glucose-3(4)-C^{14} was prepared in this laboratory according to the method of Wood et al (14).

ADMINISTRATION OF LABELED SUBSTRATES: A prescribed amount of the labeled substrate, dissolved in 0.2 ml of water, was introduced into the fruit according to the modified vacuum infiltration method described by Barbour, Buhler, and Wang (2). The procedure was repeated once again, thereby 0.2 ml of water was introduced into the fruit as flushing agent.

RADIORESPIROMETRIC EXPERIMENTS: Radiorespirometric experiments were carried out essentially in the same manner described by Barbour et al. The chemical level and the radiochemical level of the variously labeled substrates are given in table I.

EVALUATION OF TECHNIQUE FOR SUBSTRATE ADMINISTRATION: Radiorespirometric experiments were carried out with tomato fruits administered respectively with four chemical levels of glucose, namely 2.0 mg, 20.0 mg, 50.0 mg, and 100.0 mg. The radioactivity in the nature of C^{14} uniformly labeled glucose was maintained the same throughout this series.

RADIOACTIVITY ASSAY METHOD: Samples of respiratory CO_2 obtained in the radiorespirometric experiments were converted to $BaCO_3$ which were in turn mounted on aluminum planchets by the centrifugation technique and their radioactivity deter-

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[2] This research was supported in part by a contract from the Atomic Energy Commission (No. AT(45-1)-573), published with the approval of the Monographs Publications Committee, Research Paper No. 368, School of Science, Oregon State College.

[3] A portion of the work described herein is taken from the thesis presented by W. P. Doyle for the degree of Doctor of Philosophy at Oregon State College, 1960. Present address, Texaco Research Laboratories, Beacons, N. Y.

TABLE I

RADIORESPIROMETRIC STUDIES ON TOMATO FRUITS CATABOLIZING GLUCOSE-U-C^{14}, NA-PYRUVATE-C^{14}, NA-ACETATE-C^{14}, AND K-GLUCONATE-C^{14}

SUBSTRATE	RADIOCHEMICAL LEVEL, μc^*/FRUIT	CHEMICAL LEVEL, MG/FRUIT
Glucose-U-C^{14}	1.00	2.0
Glucose-U-C^{14}	1.00	20.0
Glucose-U-C^{14}	1.00	50.0
Glucose-U-C^{14}	1.00	100.0
Na-pyruvate-1-C^{14}	0.42	0.42
Na-pyruvate-2-C^{14}	0.38	0.42
Na-pyruvate-3-C^{14}	0.29	0.42
Na-acetate-1-C^{14}	0.37	0.31
Na-acetate-2-C^{14}	0.30	0.31
K-gluconate-1-C^{14}	1.19	18.6
K-gluconate-2-C^{14}	0.22	18.6
K-gluconate-3(4)-C^{14}	0.10	18.6
K-gluconate-6-C^{14}	0.19	18.6

* Counter efficiency: 1.00 μc = 3.00 $\times$ 10^5 cpm

mined with a gas flow Geiger-Müller counter equipped with a thin mylar window. Counting data were corrected for self-absorption and background in the usual manner. All radioactivity measurements were carried out to a standard deviation of 3 %.

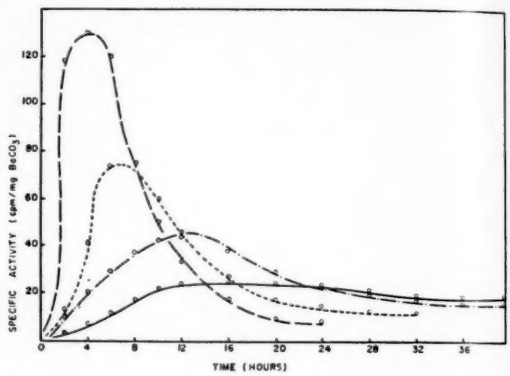
RESULTS AND DISCUSSION

Radiorespirometric data in the present report are the average of replicate experiments. The average deviation between replicates was 3 %.

EVALUATION OF TECHNIQUE FOR SUBSTRATE ADMINISTRATION: The basic concept of the radiorespirometric method in the study of carbohydrate catabolism relies on the comparative rates of radiochemical recovery in respired CO_2 from C^{14} specifically labeled substrates. It is, therefore, of great importance that the substrate levels of a given set of C^{14} specifically labeled compounds are optimal in magnitude and identical to each other within a given experiment. This implies further that the dilution of the administered substrate by the same compound of biological origin during the substrate administration process must be reasonably uniform in magnitude and rate among the experiments of a given set. This condition would be upheld if the substrate solution were administered to a defined area, such as the vascular bundle of the fruit, at the time of substrate administration provided that different fruit contained relatively consistent amounts of endogenous materials in the infiltrated area. In order to test whether or not the foregoing described conditions were satisfied when the vacuum infiltration method was used for substrate administration, an experiment employing a variable level of labeled glucose substrate was carried out. Findings of this experiment are given in figure 1.

The specific activities for the respired $C^{14}O_2$ were not directly proportional to the specific activities of the administered glucose. This is not surprising inasmuch as a sizable amount of fruit glucose is known to be present in the infiltrated area. Labeled glucose upon its administration was mixed intimately with the fruit glucose in the vascular bundle and engaged immediately in active catabolism accounting for the ascending slope of the specific activity curve.

Meanwhile, the slow diffusion of the administered glucose from the vascular bundle into other parts of



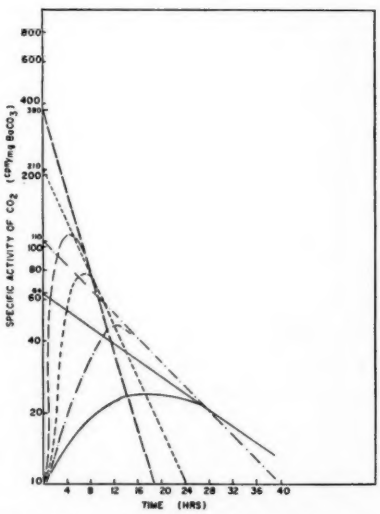


FIG. 1 (*upper*). Interval specific activity and cumulative recoveries of respired CO_2 from tomato fruits metabolizing glucose-U-C^{14}. Two milligrams of added glucose — — — —, 20 mg of added glucose - - -, 50 mg of added glucose – · – · –, 100 mg of added glucose ———.

FIG. 2 (*lower*). The extrapolation of the specific activities of $C^{14}O_2$ to the time of substrate administration. Two milligrams of added glucose —— —— ——, 20 mg of added glucose - - - -, 50 mg of added glucose – · – · –, 100 mg of added glucose ————.

the fruit together with the dilution of labeled glucose by the fruit glucose produced in a continuous turnover process soon resulting in a gradual decline of the specific activity of the respiratory $C^{14}O_2$. Inasmuch as the glucose turnover rate, presumably a relatively constant value, controls the amount of fruit glucose produced in a given time interval, one would expect the reduction of the prevailing specific activity of the labeled glucose in the vascular bundle to be less in the experiments with higher level of substrate glucose. As shown in figure 1, the peak specific activity of $C^{14}O_2$ appeared later with larger amount of glucose administered, in fact, when 100 mg of glucose was introduced into the vascular bundle, the specific activity of $C^{14}O_2$ reached a peak value at 12 hours after the substrate administration and maintained a relatively constant value throughout the experiment. The latter observation implies further that the amount of fruit glucose produced in a unit time is relatively small in comparison with the large amount of the administered glucose resulting in an insignificant dilution effect throughout the experimental duration. Bearing the above mentioned processes in mind, it is therefore possible to extrapolate the descending slope of each of the specific activity curves to the time of substrate administration to obtain the specific activities of the respiratory $C^{14}O_2$ at the time of substrate administration. Such extrapolation, expressed in semilogarithmic plots, is given in figure 2. Moreover, it appears that use can be made of the extrapolated values so obtained to calculate the amount of fruit glucose present in the immediate infiltrated area according to the principle of isotopic dilution.

The equation for estimating the amount of fruit glucose is derived as follows:

Let: S_y = the extrapolated specific activity of respiratory $C^{14}O_2$ at zero time (in cpm per mM of $BaCO_3$) when the labeled glucose (expressed as Y mM of carbon) is administered to the fruit and mixed intimately with fruit glucose (expressed as X mM of carbon).

S_z = the extrapolated specific activity of respiratory $C^{14}O_2$ at zero time when the labeled glucose (expressed as Z mM of carbon) is administered to the fruit and mixed intimately with fruit glucose (expressed as X mM of carbon).

P = reduction factor on the specific activity of $C^{14}O_2$ due to the combustion of non-labeled carbonaceous compounds at zero time. P is assumed to be a constant value for fruit of the same size, weight, and maturity. P is always greater than one.

N_o = total activity of glucose-U-C^{14} administered (in cpm).

then:

$$\frac{S_y.P}{S_z.P} = \frac{\dfrac{No}{Y + X}}{\dfrac{No}{Z + X}}$$

Solving for **X**:

$$X = \frac{S_z.Z - S_y.Y}{S_y - S_z} \qquad \text{I}$$

Results of calculations carried out according to equation I by making use of any two of the four sets of glucose levels are summarized in table II. The fact that a reasonably constant value of 17.1 ± 0.5 mg was obtained for the amount of fruit glucose present in the infiltrated area reflects the reliability of the data on the specific activities of $C^{14}O_2$. This implies that the administered labeled glucose was diluted by the prevailing fruit glucose instantaneously and evenly so that the specific activities of $C^{14}O_2$, particularly the earlier sample, bear a definite relationship to the

TABLE II

ESTIMATION OF FRUIT GLUCOSE IN VASCULAR BUNDLE OF TOMATOES

	LEVEL OF GLUCOSE ADMINISTERED (MG)/FRUIT*			
	2	*20*	*50*	*100*
Tot. radioactivity (cpm)**	3.0×10^5	3.0×10^5	3.0×10^5	3.0×10^5
Sp. act. glucose (cpm/mM glucose)	4.5×10^6	4.5×10^5	1.8×10^5	9.0×10^4
Respiratory CO_2 ($mgCO_2$/hr)	9.2	8.7	7.4	7.2
Sp. act. of $C^{14}O_2$ at zero time (fig 2)***				
cpm/mg $BaCO_3$	380	210	110	64
cpm/mM Carbon	7.50×10^4	4.10×10^4	2.12×10^4	1.23×10^4
Fruit glucose (as mM of carbon) estimated eq I for substrate levels of				
2 mg	...	0.66	0.58	0.58
20 mg	...	...	0.58	0.58
50 mg	...	...	...	0.64
100 mg	...	...	...	...

Average of estimated amount of fruit glucose = 0.57 ± 0.02 mM carbon or 17.1 ± 0.5 mg (std. dev).
* Fruit weights ranged 45 to 46 g.
** Counter efficiency 1.00 $\mu c = 3.00 \times 10^5$ cpm.
*** Average value of triplicate experiments.

amounts of glucose administered. The finding consequently provided experimental support for the feasibility of employing the vacuum infiltration technique for substrate administration in the radiorespirometric studies of glucose catabolism in tomatoes. Furthermore, the estimated magnitude of the content of the fruit glucose in the infiltrated area also lends justification to the substrate level employed by Barbour et al in their studies. This is true in view of the fact that the amount of glucose used in the experiments of Barbour et al 2.0 mg (2), should not inflict an unphysiological change in the concentration of fruit glucose in the infiltrated area.

RADIORESPIROMETRIC STUDY OF ACETATE AND PYRUVATE CATABOLISM: Buhler, Hansen, Christensen, and Wang (4) and Wang, Hansen, and Christensen (12) by means of the incorporation of C^{14}-labeled acetate, pyruvate, and CO_2 have suggested that the tricarboxylic acid (TCA) cycle may be operative in tomato fruit (4, 12). The findings of Howard and Yamaguchi, that the enzymes of the TCA cycle are present in pepper fruit (7) (also a member of the

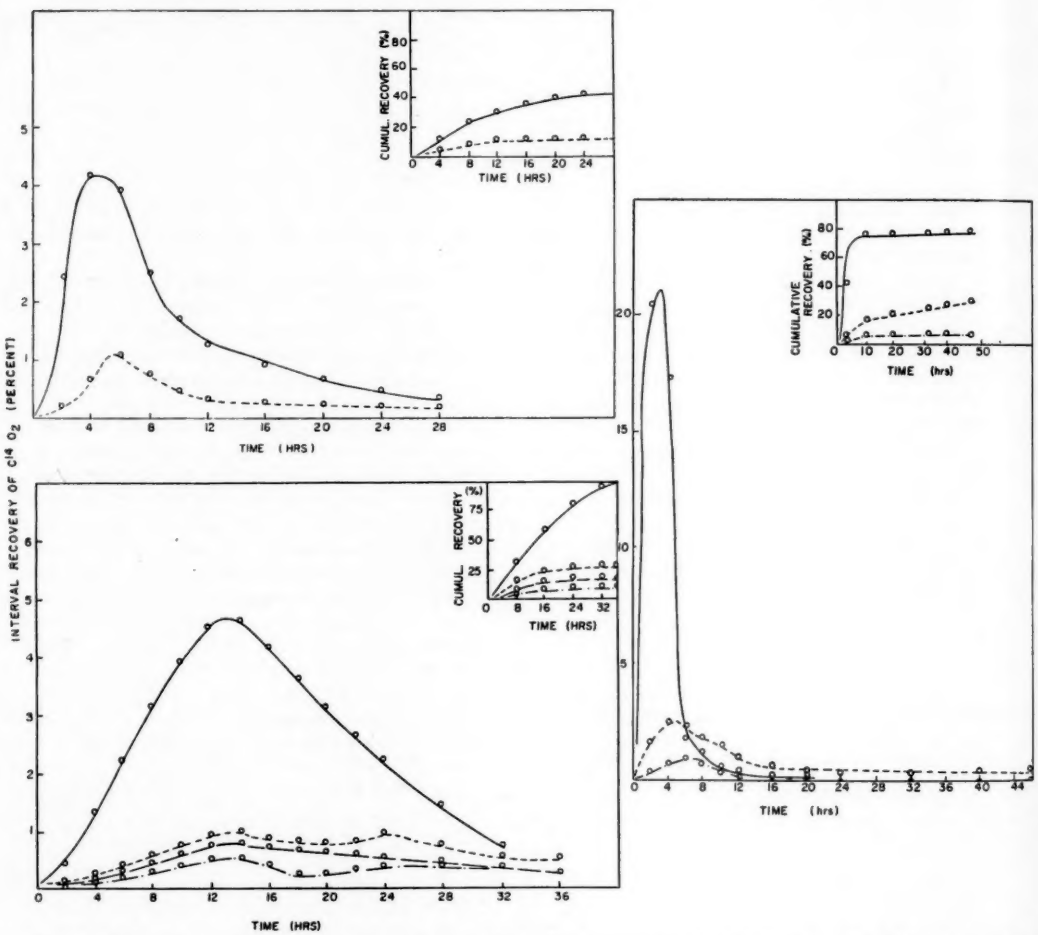


FIG. 3 (*upper left*). Interval and cumulative radiochemical recovery of administered radioactivity in respired CO_2 from tomato fruits metabolizing C^{14} specifically labeled acetate. Acetate-1-C^{14} ————. Acetate-2-C^{14} — — — — —.

FIG. 4 (*right*). Interval and cumulative radiochemical recovery of administered radioactivity in respired CO_2 from tomato fruits metabolizing C^{14} specifically labeled pyruvate. Pyruvate-1-C^{14} ————. Pyruvate-2-C^{14} — — — — Pyruvate-3-C^{14} - · - · -.

FIG. 5 (*lower left*). Interval and cumulative radiochemical recovery of administered radioactivity in respired CO_2 from tomato fruits metabolizing C^{14} specifically labeled gluconate. Gluconate-1-C^{14} ————. Gluconate-2-C^{14} —— —— ——. Gluconate-3,4-C^{14} - - - - - . Gluconate-6-C^{14} -·-·-.

Solanacea) give additional support to the function of the TCA cycle in fruit of this family.

To examine the extent of the operation of the TCA cycle in tomatoes, and to test the assumption made by Barbour et al (2) that the decarboxylation of pyruvate derived from triose in the EMP pathway is prompt and extensive, additional radiorespirometric experiments were carried out. Acetate-1- and -2-C^{14} and pyruvate-1-, -2- and -3-C^{14} were used as substrates. The interval and cumulative radiochemical recoveries of substrate activity in the respired CO_2 from tomatoes metabolizing these substrates are shown in figures 3 and 4. The rate of appearance of C-1 of pyruvate in respired CO_2 is extremely rapid and total recovery from C-1 is 78 % at 10 hours after substrate administration. Since the labeled pyruvate has to be taken up by the fruit cells prior to any metabolic processes and since some of the administered pyruvate may have diffused into areas of relatively low metabolic activity, it can be concluded that the original assumption of Barbour et al, in connection with the extensive pyruvate decarboxylation in tomatoes is justifiable. Interesting information is also revealed concerning the biosynthetic role of the TCA cycle by examining the relative extents of oxidation of the carboxyl (C–1) and methyl (C–2) carbon atoms of acetate and the carbonyl (C–2) and methyl (C–3) carbon atoms of pyruvate. Thus, the ratio of cumulative radiochemical recovery in CO_2 from C-1 to that from C-2 of acetate at 28 hours (when $C^{14}O_2$ evolution had become quiescent) is 0.41/0.11 or 3.7/1, a value which compares favorably with the analogous ratio for pyruvate (C–2/C–3), 0.24/0.07 or 3.5/1. The fact that these ratios are in good agreement with one another, suggests strongly that pyruvate is indeed catabolized by way of an oxidative decarboxylation giving rise to acetyl-coenzyme A which in turn is oxidized via the TCA cyclic processes. Magnitudes of the observed ratios, meanwhile, indicate that the methyl carbon atoms of acetate are preferentially conserved in the TCA cycle processes; presumably this is the result of drainage of various intermediates for concurrent biosynthetic processes.

Radiorespirometric Study of Gluconate Catabolism: In the radiorespirometric method for the estimation of pathway participation in glucose catabolism, it was assumed that the decarboxylation of phosphogluconic acid was a rapid and extensive process. To test the validity of this assumption and to trace the fate of the pentose formed via the decarboxylation of phosphogluconic acid in tomato metabolism, a series of radiorespirometric experiments, employing gluconate-1-, -2-, -3(4)-, and -6-C^{14} as test substrates, was carried out. The interval and cumulative radiochemical recoveries of substrate activity in respired CO_2 are given in figure 5.

The order of radiochemical recoveries of the carbon atoms of gluconate in CO_2 is C-1 > C-3(4) > C-2 > C-6. The recovery from C-1 is practically quantitative, whereas the remaining carbon atoms are considerably conserved in the respiratory activities. These findings thus render experimental support to the assumption under consideration. It is of interest to also examine the observed order of radiochemical recoveries in CO_2 in light of the known pathways for pentose metabolism. Apparently the findings cannot be explained solely on the basis of a pathway involving the repeated cycling of pentose phosphate by way of the pentose phosphate cycle; such a mechanism would produce the relative order of radiochemical recoveries in CO_2 from carbon atoms of gluconate C-1 > C-2 > C-3(4) > C-6 or at best C-1 > C-2 = C-3(4) > C-6. On the other hand the data may be readily interpreted if one takes into consideration the sequential operation of the 6-phosphogluconate decarboxylation reaction, the pentose phosphate cycle reactions, and catabolism of the reformed hexose phosphate via mainly the Embden-Meyerhof-Parnas pathway (EMP) (2). The sequence would result in the conversion of the pentose phosphate, as derived from gluconate, to hexose phosphate. This in turn may be metabolized mainly via the EMP pathway.

The origin of the carbon atoms of glucose can be traced to gluconate according to the scheme described by Beevers (3) if one ignores the possible interferences from the operation of such side reactions as exchange reactions involving triose isomerase and transaldolase reactions (9, 15) and randomization via reversible transketolase and transaldolase reactions. According to Beever's scheme, one would expect to find 2/3rds of the radioactivity originally in C-2 of gluconate relocated in the C-1 position and the remainder in the C-3 position of reformed hexose phosphate. Similarly, one would expect to find 2/3rds of C-3, 4 radioactivity of gluconate relocated in C-3, 4 of the hexose and 1/3rd in the C-2 position. The radioactivity originally in C-6 of gluconate would be relocated to the extent of 5/6ths in C-6 and 1/6th in C-1 of the hexose phosphate. By taking into consideration the pathway participation estimated by Barbour et al (2), namely 16 % of the catabolized glucose is degraded by way of the hexose monophosphate pathway and 84 % via the EMP pathway, it is reasonable to expect a greater radiochemical recovery from gluconate-3(4)-C^{14} in CO_2 than from gluconate-2-C^{14} as observed in the present experiment. Alternately, the observed order of radiochemical recoveries may also be interpreted as being the result of a C_2-C_3 cleavage of the pentose, giving rise to glycolic acid and glyceraldehyde (10). The glycolic acid formed could then be oxidized to glyoxylic acid which would result in a preferential conversion of the carboxyl carbon atom (equivalent to C–3 of gluconate) to CO_2 as compared to the aldehyde carbon atom (equivalent to C-2 of gluconate). The nature of the oxidation of glyoxylate to CO_2 has been studied by Doyle et al (5). It is possible that the glyceraldehyde formed in such a C_2-C_3 cleavage could be oxidized to pyruvate, of which the carboxyl carbon atom (equivalent to C–4 of gluconate) would be nearly quantitatively oxidized to CO_2 as demonstrated in the radiorespirometric studies of pyruvate utilization. The combined effect of the operation of these two reaction sequences

is again, a preferential conversion of C-3(4) of gluconate to metabolic CO_2 as compared to that of C-2.

Further experiments are now being carried out to determine the exact fate of the pentose derived in the hexose monophosphate pathway.

Summary

The amount of fruit glucose in the immediate infiltrated area when C^{14}-labeled glucose is administered into tomato by means of the vacuum infiltration technique has been determined by the isotopic dilution method. It appears that the substrate glucose was infiltrated into a well defined area in the fruit. Several key assumptions necessary for deriving the equation for the estimation of glucose catabolism in tomato have been verified by a series of radiorespirometric experiments using C^{14} specifically labeled acetate, pyruvate and gluconate as test substrates.

Literature Cited

1. Axelrod, B. and H. Beevers. 1956. Mechanism of carbohydrate breakdown in plants. Ann. Rev. Plant Physiol. 7: 267–298.
2. Barbour, R., D. R. Buhler, and C. H. Wang. 1958. Identification and estimation of catabolic pathways of glucose in fruits. Plant Physiol. 33: 396–400.
3. Beevers, H. 1957. Intermediates of the pentose phosphate pathways as respiratory substrates. Plant Physiol. 31: 418–423.
4. Buhler, D., E. Hansen, B. E. Christensen, and C. H. Wang. 1956. The conversion of $C^{14}O_2$ and $CH_3C^{14}OCOOH$ to citric and malic acids in tomato fruit. Plant Physiol. 31: 192–195.
5. Doyle, W. P., R. Huff, and C. H. Wang. 1960. Role of glyoxylate in biosynthesis of acids in tomato fruit. Plant Physiol. 35: 745–750.
6. Gibbs, M., and H. Beevers. 1955. Glucose dissimulation in the higher plant. Effect of age of tissue. Plant Physiol. 30: 343–347.
7. Howard, F. D. and M. Yamaguchi. 1957. Respiration and the oxidative activity of particulate fractions from developing pepper fruits (*Capsicum annum* L.). Plant Physiol. 32: 418–423.
8. Moore, S., and K. P. Link. 1940. Carbohydrate characterization. Jour. Biol. Chem. 133: 293–311.
9. Noble, E. P., R. L. Stjernholm, and A. S. Weisberger. 1959. Distribution of C^{14} in glycogen glucose of rabbit polymorphonuclear leucocytes. Federation Proc. 18: 1164.
10. Rabson, R., and N. E. Tolbert. 1957. Biosynthesis of serine in plants. Plant Physiol. suppl. 32: xxxv.
11. Silva, G. M., W. P. Doyle, and C. H. Wang. 1958. Glucose catabolism in the American cockroach. Nature 182: 102–104.
12. Wang, C. H., E. Hansen, and B. E. Christensen. 1953. Conversion of C^{14} labeled acetate to citric and malic acid in tomato fruit. Plant Physiol. 28: 741–745.
13. Wang, C. H., I. Stern, C. M. Gilmour, S. Klungsoyr, D. J. Reed, J. J. Bialy, B. E. Christensen, and V. H. Cheldelin. 1958. Comparative study of glucose catabolism by the radiorespirometric method. Jour. Bact. 76: 207–216.
14. Wood, H. G., N. Lifson, and V. Lorber. 1945. The position of fixed carbon in glucose from rat liver glycogen. Jour. Biol. Chem. 159: 475–489.
15. Wood, H. G., L. Ljungdahl, D. Coun, and E. Racker. 1959. Transketolase and tracer patterns of hexoses. Federation Proc. 18: 1403.

STUDIES ON PYRUVATE METABOLISM AND CYTOCHROME SYSTEM IN NEUROSPORA TETRASPERMA[1, 2]

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Although it has been known for more than 30 years that dormancy in Neurospora ascospores can be broken by heat treatment (30), the mechanism is not yet understood. Goddard (10) concluded from his studies that the metabolic block overcome by the heat treatment was concerned with the enzyme carboxylase. Studies of Sussman et al (36) demonstrated that both the apoenzyme and coenzyme were present in dormant and heat-activated ascospores in nearly equivalent amounts. They concluded that activation induces the formation of an oxidative system, possibly operating through the TCA cycle, that is different from that in the dormant ascospores. Recent work from this laboratory (20, 38) on the nature of the endogenous substrates in dormant and activated ascospores supports this idea of a qualitative change in metabolism after activation.

Because pyruvate appeared to be a key intermediate in the suggested development of an oxidative system, it was thought that pyruvic oxidase might be present in the activated ascospores but not in the dormant ascospores. Since anions of the size of pyruvate do not penetrate dormant spores (37), it is necessary to study pyruvate oxidation in cell-free extracts. The studies reported here were originally undertaken in an attempt to demonstrate the presence of this enzyme system in cell-free extracts of the ascospores and in other stages of the life cycle including conidia and mycelia of *Neurospora tetrasperma*. A preliminary report of this work has been published (16).

MATERIALS AND METHODS

Ascospores of *Neurospora tetrasperma* Shear and Dodge were produced from a cross of strains 394.4 and 394.5 originally obtained by Dr. A. S. Sussman from Dr. B. O. Dodge. The ascospores were grown and harvested using the procedure of Goddard (9) and freed of contaminant conidia by the technique of Sussman (35). Heat activation was accomplished by placing a suspension of ascospores in a centrifuge tube in a 58° C water bath for 10 minutes.

Good mycelial growth in liquid shake culture was obtained by inoculation of a suspension of conidia of strain 394.5 into the complete medium of Horowitz [cited by Ryan (26)]. Sixty-five ml of the medium were added to 250 ml Erlenmeyer flasks, inoculated with 5 ml of a conidial suspension and incubated on a reciprocal shaker at 21° C. The mycelium was harvested by filtration on a suction funnel and the mycelial mat obtained was washed with distilled water and used for enzyme preparations. In determining dry weights for the growth curve of the mycelium, the flask contents were washed into a tared, 25 ml, coarse porosity, fritted glass Buchner funnel, washed, and the mat and funnel dried to constant weight at 105° C.

Conidia were produced on 50 ml of Horowitz's medium solidified with 1.5 % agar in wide-mouth 500 ml Erlenmeyer flasks. After inoculation of the media with a suspension of strain 394.5 conidia, the fungus was allowed to grow for 2 days at 21° C under approximately 500 foot-candles of light. Conidia formation was stimulated by removal of the cotton plugs overnight (23). The conidia were harvested with distilled water and passed through glass wool in a Gooch crucible to remove mycelial fragments (26). The resulting suspension of orange conidia was washed several times in distilled water by centrifugation.

Gas exchange was measured with a conventional Warburg apparatus using standard techniques and a 26° C water bath. Gas exchange was placed on a nitrogen basis by determining the nesslerizable nitrogen present in an aliquot of the cell-free extract after digestion with 1 : 1 concentrated sulfuric acid (17).

Acetaldehyde analyses were carried out by the method of Stotz (33). A slow stream of air was passed through the Warburg flask in which the reaction was carried out and the effluent containing acetaldehyde was trapped in 6 ml of cold, freshly prepared 2 % sodium bisulfite. This bisulfite solution was used directly for the acetaldehyde determinations using paraldehyde for preparation of the standard curve.

Cell-free extracts of ascospores are difficult to obtain because of the ascospores' exceedingly tough coat and the procedure used did not bring about breakage of all spores present. Thirty to 75 mg of dormant or activated ascospores were added to the mortar of a Ten Broeck tissue homogenizer. Three or 4 ml of 0.05 M phosphate buffer pH 6.0 or other homogenizing medium, were added and the teflon pestle attached to a stirring motor. The motor was

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[2] Most of the data reported here were taken from a thesis submitted to the Horace H. Rackham School of Graduate Studies of The University of Michigan in partial fulfillment of requirements for the degree of Doctor of Philosophy, 1958.

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started and the cup raised and lowered around the pestle so that the spores were constantly agitated vertically as well as horizontally. After 10 minutes of homogenization, the unbroken spores were centrifuged off at 800 × G and the operation repeated. The supernatants were combined to give a faintly yellow and turbid cell-free preparation. All operations were carried out at 4° C.

The mycelial mat was easily homogenized in a Servall High Speed Omni-Mixer with a top-drive stainless steel rotor and a 50 ml stainless steel cup. The mat (approx. 0.5 gm dry wt) and 15 ml "Superbrite" glass beads, grade No. 110 (Minnesota Mining and Manufacturing Co.) were suspended in 20 ml 0.05 M phosphate buffer pH 6.0 precooled in an ice bath. Homogenization was accomplished in 1 minute at a rotor speed of 14,000 rpm and the resulting homogenate was centrifuged at 800 × G for 20 minutes at 4° C to give a colorless, turbid cell-free extract.

Conidia were also homogenized in the Servall Omni-Mixer. They were suspended in about 10 ml of 0.06 M phosphate buffer at pH 6.1 and enough fine glass beads were added to form a slurry. After homogenization and centrifugation, an orange cell-free extract was obtained.

In some experiments, the cell-free extract was fractionated by centrifugation in a Servall Angle Centrifuge, Model SS-1, in a 4° C room for 10 minutes at 16,000 × G.

Cytochrome oxidase activity in cell-free extracts was determined by both manometric and spectrophotometric means. The manometric technique used was that of Fritz and Beevers (8) with *p*-phenylenediamine (PPD) as the reducing agent which Slater (31) found to be effective in reducing both endogenous and exogenous cytochrome c. The PPD was added at zero time and readings were made every 5 minutes for 30 minutes. The initial rate of oxygen uptake was estimated graphically and autoxidation rates of cytochrome c were estimated and subtracted from the observed rates (31). In the spectrophotometric method used, the decrease in optical density at 550 mμ due to oxidation of the cytochrome c (Sigma Chemical Co.) was followed in a Beckman Model DU spectrophotometer (6, 8).

Slater (31) showed that for the manometric method, the rate of oxygen uptake depended not only on the cytochrome oxidase activity but also on the concentration of cytochrome c. Thus, to determine the maximal activity of cytochrome oxidase by both procedures, it was necessary to measure the rate of oxidation at different cytochrome c concentrations and to extrapolate to infinite substrate concentration. For the extrapolation the method of Lineweaver and Burk (19) was used. The theoretical maximum velocity of the enzyme action is expressed as the V_{max} and the values obtained in the spectrophotometric method were converted to the units used in the manometric method by use of the equation derived by Fritz and Beevers (8).

The absorption bands of the cytochromes were observed with a Zeiss pupillary spectroscope mounted on a microscope as described by Hartree (13). Mycelial extracts were prepared using the procedure of Boulter and Derbyshire (4). In order to get enough particulate material to observe cytochrome bands, at least 10 g (wet wt) of mycelia were required. For the observations, the particles in buffer were packed into small cells and the cytochromes reduced with sodium hydrosulfite. Quantitative measurements were made using the wedge trough procedure of Hartree (13).

Results

Carboxylase: It has been earlier shown by Sussman et al (36) that the carboxylase content of cell-free extracts of ascospores did not change quantitatively after activation. The presence of carboxylase in these tissues was confirmed in the present work and carboxylase shown to be present in the mycelium also. In all experiments, approximately 90 % of the stoichiometric amount of carbon dioxide was formed from the pyruvate added whether the experiment was carried out aerobically or anaerobically.

Evidence that acetaldehyde is produced from pyruvate by carboxylase in the mycelial extracts is presented in table I. In flasks 16 and 32 after carbon dioxide evolution in the flasks was essentially complete, air was passed through the flasks and into a trap containing cold 2 % sodium bisulfite for 50 minutes. Analysis of the bisulfite solution for acetaldehyde showed that on a molar basis approximately half the pyruvate was converted to acetaldehyde. In flask 37 air was passed through the flask and into a bisulfite trap throughout the experiment and on a molar basis all of the pyruvate added could be accounted for as acetaldehyde.

Pyruvic Oxidase System: Several different procedures were tried in attempts to demonstrate the presence of a pyruvic oxidase system in different stages of the life cycle of Neurospora. The general procedures followed were those of Walker and Beevers (40) and Lieberman and Biale (18), both of

Table I

Production of Acetaldehyde by Extracts of Mycelia of N. Tetrasperma

Flask No.	Sodium pyruvate added μM	Acetaldehyde produced μM	% Theory
16*	1.98	1.08	55
32*	1.98	0.92	46
37**	1.98	2.04	103

* Air passed through flask and $NaHSO_3$ trap after carbon dioxide evolution was essentially complete (86 % of stoichiometric amount of carbon dioxide obtained).

** Air passed through flask and $NaHSO_3$ trap throughout course of experiment.

whom used particulates isolated from higher plant tissue. Phosphate buffers of pH 6.0 and 7.4, and 0.5 M sucrose solutions were used in the enzyme extraction procedures. A number of different substances tried in various combinations failed to stimulate oxygen uptake in the presence of pyruvate. These included yeast extract, liver extract, diphosphopyridine nucleotide, adenosine triphosphate, adenosine monophosphate, cytochrome c, diphosphothiamine, coenzyme A, glucose, Mg^{++}, flavine mononucleotide, 2, 4-dinitrophenol, succinate, malate, fumarate, and oxalacetate. Both the whole brei and the particle fraction were used, as well as particles treated with deoxycholate, which removes cytochrome c (2) and probably much lipid material, since fatty acids have been shown by Scholefield (27) to inhibit pyruvate oxidation in rat kidney mitochondria.

PYRUVATE AND *p*-PHENYLENEDIAMINE: Owens (24) reported that he was unable to obtain pyruvate oxidation with cell-free extracts of the conidia of *Neurospora sitophila* until he added *p*-phenylenedia-

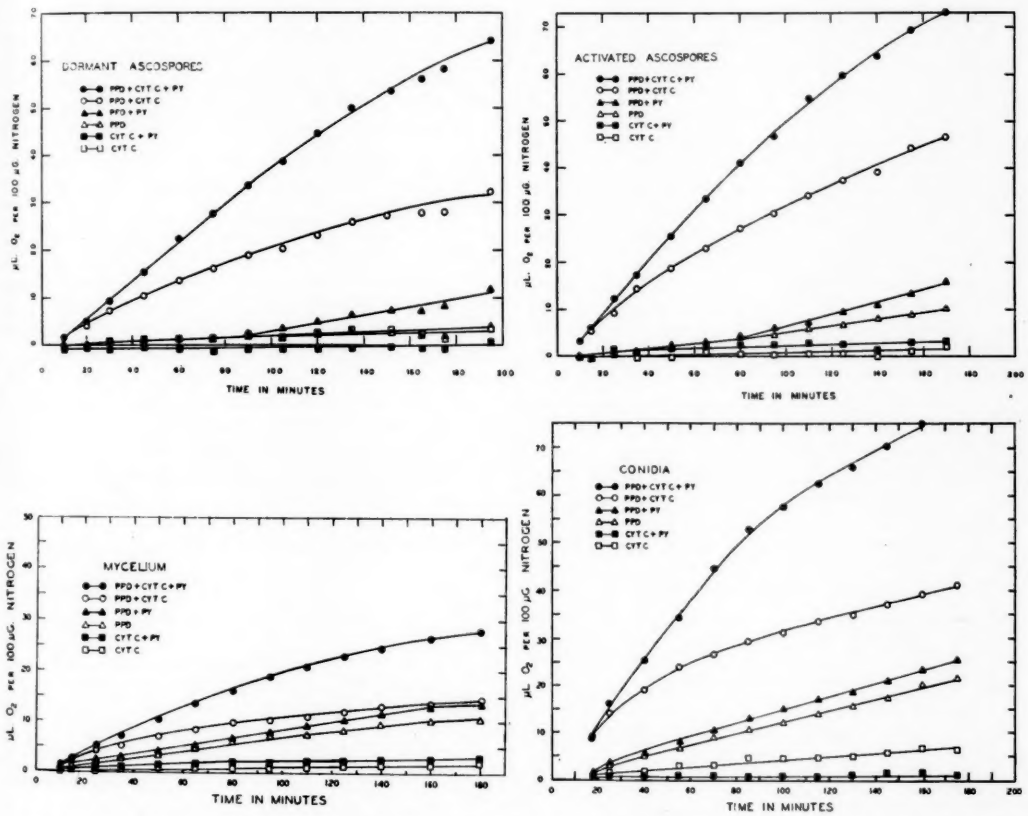


FIG. 1 (*upper left*). Oxygen uptake by dormant ascospore extracts with added pyruvate, PPD, and cytochrome c. Each vessel contained 0.5 ml cell-free extract which contained 107 μg nitrogen in phosphate buffer at pH 6.0, giving a final concentration of 0.05 M. Addenda were 0.1 ml sodium pyruvate (10 μM), 0.1 ml PPD (10 μM), 0.05 ml cytochrome c (0.03 μM), and water to total 1.0 ml. If a component was omitted, an equal volume of water was substituted. The center well contained 0.2 ml 20% potassium hydroxide.

FIG. 2 (*upper right*). Oxygen uptake by activated ascospore extracts with added pyruvate, PPD, and cytochrome c. Each vessel contained 10.5 ml cell-free extract of ascospores incubated for 2 hours containing 92 μg nitrogen in phosphate buffer at pH 6.0, giving a final concentration of 0.05 M. Addenda were the same as in figure 1.

FIG. 3 (*lower left*). Oxygen uptake by mycelial extracts with added pyruvate, PPD, and cytochrome c. Each vessel contained 0.5 ml cell-free extract of a 2½-day-old mycelial culture containing 352 μg nitrogen in phosphate buffer at pH 6.0, giving a final concentration of 0.05 M. Addenda were the same as in figure 1.

FIG. 4 (*lower right*). Oxygen uptake by conidial extracts with added pyruvate, PPD, and cytochrome c. Each vessel contained 0.5 ml cell-free extract containing 68 μg nitrogen in phosphate buffer at pH 6.1, giving a final concentration of 0.03 M. Addenda were the same as in figure 1.

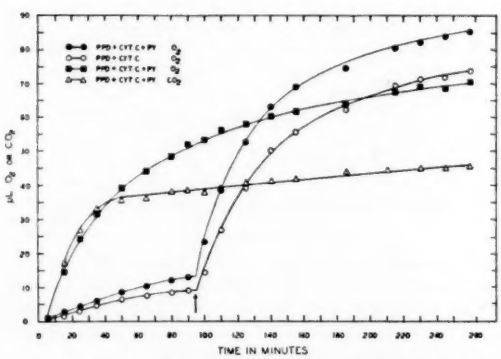
mine (PPD) with the pyruvate. He also noted that added cytochrome c increased the oxygen uptake further. Therefore, experiments were carried out to determine if the same pyruvate oxidation system was present in extracts of *N. tetrasperma*. These experiments were carried out with extracts of dormant and activated ascospores, mycelium, and conidia of *N. tetrasperma*. In all cases, it was found that if pyruvate was added with cytochrome c and PPD, it stimulated oxygen uptake over that found with the cytochrome c and PPD alone. Results of these experiments are shown in figures 1, 2, 3, and 4.

None of the extracts showed significant oxygen uptake in the presence of pyruvate plus cytochrome c or with cytochrome c alone. With PPD alone, the dormant spore extract showed no oxygen uptake but the activated one did consume a small amount of oxygen. In the mycelial and conidial extracts, there was a considerable amount of oxygen uptake with PPD alone. In all cases, the oxygen uptake with PPD was enhanced when pyruvate was added. All extracts showed considerable oxygen uptake in the presence of PPD and cytochrome c, in contrast to Owens' (24) results with conidial extracts. Oxygen uptake in the presence of cytochrome c and PPD is generally attributed to cytochrome oxidase and on this basis the oxygen uptake in this experiment is ascribed to this enzyme. Adding pyruvate always enhanced oxygen uptake over those systems with only PPD and cytochrome c added but the added uptake was never more than 10 to 15 % of the theoretical expected for the complete oxidation of pyruvate.

Several conclusions can be derived from these data. On a nitrogen basis, the mycelial extract was generally less active than those of the ascospores and conidia whose specific activities were almost equivalent. An important difference involved the uptake of oxygen in the presence of PPD alone. While this was very low with the ascospore extracts, it was nearly as great as that of the cytochrome oxidase assay system in mycelial extracts. In this respect the conidia were intermediate. This aspect of the comparative metabolism of the cell-free extracts of *Neurospora tetrasperma* was studied further and the results are reported below.

PPD-Stimulated Pyruvate Oxidation: Essentially stoichiometric carbon dioxide production, lack of oxygen uptake, and acetaldehyde formation by the action of Neurospora extracts on pyruvate suggested that the principal enzyme in this extract acting on pyruvate was carboxylase. In an effort to better understand the reason for oxygen uptake when PPD was added with pyruvate, several experiments were carried out.

Owens (24) suggested that the PPD-stimulated oxidation of pyruvate might be independent of the decarboxylation and that the product of pyruvate decarboxylation might be the compound being oxidized. To determine if this suggestion might be correct, pyruvate was tipped into a Warburg flask from the sidearm before and after stoichiometric carbon dioxide evolution was obtained. In figure 5, it can be seen that oxygen uptake did occur after pyruvate had been almost completely decarboxylated suggesting the decarboxylation product and not pyruvate was being



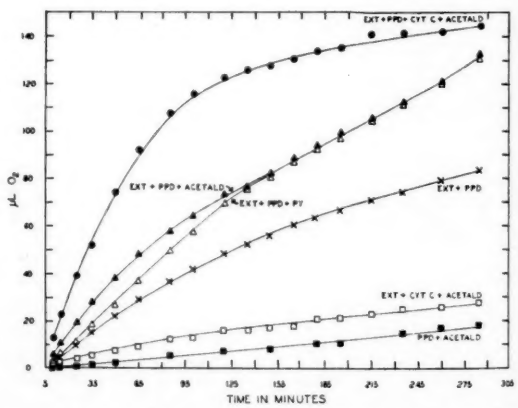


Fig. 5 (*left*). Effect of time of addition of *p*-phenylenediamine on oxygen uptake in the presence of pyruvate. In the two lower curves, the PPD was tipped in at the arrow; in the other curves, the PPD was tipped in at zero time. The vessels contained 0.5 ml of a cell-free extract of mycelium suspended in 0.045 M phosphate buffer at pH 6.1. Addenda were 0.1 ml cytochrome c (0.03 μM), 0.2 ml PPD (recrystallized, 4.4 μM), 0.2 ml sodium pyruvate (3.8 μM), and buffer to give 1.0 ml.

Fig. 6 (*right*). Oxygen uptake in the presence of *p*-phenylenediamine and pyruvate or acetaldehyde. Each vessel contained 0.5 ml of a cell-free extract of mycelium suspended in 0.045 M phosphate buffer at pH 6.1. Addenda were 0.1 ml cytochrome c (0.03 μM), 0.2 ml PPD (recrystallized, 9.3 μM), 0.2 ml sodium pyruvate (10.3 μM), and 0.1 ml acetaldehyde (10.0 μM), and buffer to total 1.0 ml.

oxidized. The decarboxylation product, acetaldehyde, was added in the presence of PPD and found to behave identically to pyruvate (fig 6) after the first 2 hours. During the first 2 hours the greater oxygen uptake with acetaldehyde and PPD over pyruvate and PPD is expected if the pyruvate must first be decarboxylated before oxygen uptake can occur.

That this is an enzymatic oxidation is suggested in the failure of boiled enzyme to stimulate oxygen uptake when added to PPD and acetaldehyde. There was no appreciable oxygen uptake with PPD and acetaldehyde with or without cytochrome c in the absence of enzyme. A PPD solution autoxidized in air or an enzymatically oxidized PPD solution from which the enzyme was removed did not stimulate oxygen uptake when added to acetaldehyde. Neither sodium 2, 6-dichlorophenolindophenol, potassium ferricyanide nor ferrocyanide, nor reduced DPN would substitute for PPD.

ENDOGENOUS CYTOCHROME C: It was noted above that oxygen uptake occurred when PPD alone was added to conidial and mycelial extracts (fig 3 and 4) but not when PPD was added to ascospore extracts (figs 1 and 2). This oxygen uptake could be due to one, or a combination, of two entirely different oxidative systems: A: laccase, an oxidase that oxidizes PPD directly, or B: cytochrome oxidase, an enzyme that oxidizes cytochrome c which is, in turn, reduced by PPD. Several experiments were carried out to determine which system was operating here.

Because laccase in other organisms has been found in the supernatant while cytochrome oxidase is in the particles, fractionation was used as a method of separating the two activities. Centrifugation of the extracts at 16,000 × G yielded particulate and supernatant fractions. The first experiment showed that 56 % of the total PPD-oxidase activity was in the particulate fraction, after centrifugation at 16,000 × G, of the extract of a 61-hour-old mycelial culture while a second showed 68 % in the same fraction of an extract of a 40-hour-old culture. Because a majority of the PPD-oxidase activity was localized in the particles, endogenous cytochrome c could be implicated in part of the oxygen uptake with added PPD.

Ball and Cooper (2) showed that in the particulates isolated from heart muscle, 95 % of the PPD-oxidase activity could be removed by homogenization of the particulate material in buffer containing 0.5 % deoxycholate. The treated particles regained their ability to oxidize PPD when cytochrome c was added. This technique was applied to particulate extracts of Neurospora mycelia in two ways. In several experiments, after the initial centrifugation of the cell-free extract, the particles were homogenized and resuspended in phosphate buffer with 0.1 % or 0.5 % deoxycholate in this buffer. They were then centrifuged again for 10 minutes and this time resuspended in deoxycholate-free buffer. The treated particles that came down during this centrifugation had a different appearance from the untreated ones in that the pellet formed was smaller and more compact. The supernatant from the treated particles was turbid while that from the untreated was clear. As shown in table II, this procedure removed most of the PPD-oxidase activity. An increase in cytochrome oxidase activity was obtained by adding cytochrome c but the total cytochrome oxidase activity of the untreated extracts was not recovered.

TABLE II

TREATMENT OF MYCELIAL PARTICLES FROM N. TETRASPERMA WITH DEOXYCHOLATE (DOC) AND RESUSPENSION IN PHOSPHATE BUFFER

CYTOCHROME C CONC	μL O_2 UPTAKE/HR*			
	0.1 % DOC		0.5 % DOC	
	TREATED	UNTREATED	TREATED	UNTREATED
0	8	34	1	15
1×10^{-5} M	...	...	26	178
1.2×10^{-4} M	67	163	...	...

* All rates are the initial rates obtained after tipping the PPD (10 μM). Extracts were prepared from a 36-hour-old mycelial culture for the 0.1 % DOC treatment and from a 37-hour-old mycelial culture for the 0.5 % DOC treatment.

In an attempt to recover maximal cytochrome oxidase activity, a second procedure was tried utilizing the particles obtained after a single 15 minute centrifugation. After resuspension in buffer plus deoxycholate, the particles were used directly. Table III shows that all the cytochrome oxidase activity could be recovered by adding cytochrome c but complete removal of cytochrome c from the particles was not effected.

TABLE III

TREATMENT OF MYCELIAL PARTICLES FROM N. TETRASPERMA WITH DEOXYCHOLATE (DOC) AND RESUSPENSION IN DOC

CYTOCHROME C CONC	μL O_2 UPTAKE/HR*			
	0.2 % DOC		0.5 % DOC	
	TREATED	UNTREATED	TREATED	UNTREATED
0	9	33	9	26
3.9×10^{-6} M	66	...	...	...
4.5×10^{-6} M	...	...	79	...
1.6×10^{-5} M	188	222	...	...
1.8×10^{-5} M	...	...	165	195
1.6×10^{-4} M	224	...	...	...
1.8×10^{-4} M	...	...	170	...
2.8×10^{-4} M	210	...	...	...
3.1×10^{-4} M	...	...	157	...

* All rates are the initial rates obtained after tipping in the PPD (35 μM). Extracts were prepared in glycylglycine buffer from a 67-hour-old mycelial culture for the 0.2 % DOC treatment and in phosphate buffer from a 36-hour-old mycelial culture for the 0.5 % DOC treatment.

From the evidence obtained by this technique, it appeared that the deoxycholate treatment did remove endogenous cytochrome c from the particles and that PPD oxidation depended upon the presence of cytochrome c in the mycelial extracts as in the heart muscle preparation of Ball and Cooper (*2*).

The manometric and spectrophotometric methods for cytochrome oxidase are based on different principles, as mentioned earlier. If laccase is present together with cytochrome oxidase, it can be assayed in the manometric method because any oxygen uptake in the presence of PPD and cytochrome c would be detected. Such activity could be due to either cytochrome oxidase, laccase or their combination. The spectrophotometric method, on the other hand, assays only cytochrome oxidase activity because the disappearance of reduced cytochrome c is followed directly. Therefore, if laccase were present with cytochrome oxidase, the manometric procedure would give higher values than the spectrophotometric, while if laccase were absent the two methods should give equivalent results.

In order to compare the two assay methods, the results must be expressed in common units, therefore the V_{max}, in terms of μl oxygen uptake per hour per mg nitrogen in the enzyme solution was used. Values for a number of these analyses are given in table IV. These data show that for the mycelial extracts (brei), the manometric and spectrophotometric results agree quite well for the first four samples. In the last four samples, the agreement is not as good, but the activities measured in the spectrophotometer were greater than those obtained manometrically. Inasmuch as the reverse would be expected if more than one oxidase were present, these data are also compatible with the notion that laccase was absent.

Consequently, data obtained by three different methods suggest that the ability of mycelial extracts to oxidize PPD without added cytochrome c is not due to laccase but to cytochrome oxidase which oxidizes reduced endogenous cytochrome c. The restricted ability of extracts of dormant and activated ascospores to carry out the oxidation of PPD suggests that they lack endogenous cytochrome c, or have only small amounts of it.

TABLE IV

CYTOCHROME OXIDASE IN EXTRACTS OF N. TETRASPERMA

TISSUE	AGE (HRS)	FRACTION	PROCEDURE: MANOMETRIC V_{max}*	PROCEDURE: SPECTROPHOTOMETRIC V_{max}*
Dormant ascospores		Brei	1,230	1,230
Dormant ascospores		Brei	1,100	940
Mycelium	43	Brei	330	...
Mycelium	47	Brei	...	280
Mycelium	64	Brei	730	820
Mycelium	66	Brei	380	400
Mycelium	68	Brei	640	1,390
Mycelium	70	Brei	410	920
Mycelium	89	Brei	340	830
Mycelium	43	Particles	3,120	...
Mycelium	47	Particles	3,590	2,950
Mycelium	64	Particles	4,830	5,950
Mycelium	66	Particles	5,150	4,150
Mycelium	64	Supernatant	140	80
Mycelium	66	Supernatant	220	140

* V_{max} values are expressed in μl O_2 uptake per hour per mg N.

TABLE V

MEASUREMENTS OF CYTOCHROME C CONTENT OF N. TETRASPERMA

SOURCE OF PARTICLES	N CONTENT μG	CYTOCHROME C MOLARITY	CYTOCHROME C μM/μG N
Dormant ascospores	25	...*	...
26-hour-old mycelium	1410	2.77×10^{-5}	5.90×10^{-6}
45-hour-old mycelium	1375	4.87×10^{-6}	1.04×10^{-6}

* No cytochrome c was detected. The lowest concentration of the standard cytochrome c solution that could be detected was 4.78×10^{-7} M.

Final evidence of the presence of cytochrome c in mycelial extracts was obtained by direct observation of particulate preparations with the spectroscope. Qualitative observations at room temperature showed bands at 550, 565, and 610 mμ presumed to be the cα, bβ, and a-a_3 cytochrome bands, respectively. These were observed in particles from both 26-hour-old mycelium and 45-hour-old mycelium. In addition, a diffuse band at 520 to 530 mμ was observed in the older mycelial particles while in the younger particles an unidentified pigment obscured this portion of the spectrum. No differences were observed when the samples were frozen in liquid air except that the diffuse band at 520 to 530 mμ was resolved into two distinct lines in that region. Quantitative measurements of cytochrome c by means of the wedge trough method of Hartree (13) are shown in table V.

Although no evidence of cytochrome c was observed in the particles from the dormant ascospores, this extract had such a low nitrogen content compared with that of the particles from the mycelia that the result was not unexpected. Because of the difficulties of obtaining ascospores in the quantities necessary to obtain an extract with a nitrogen content comparable to that of the mycelial extracts, no further experiments were carried out with this material.

CYTOCHROME OXIDASE: Table IV also affords information on the relative cytochrome oxidase activities at different phases during the growth of Neurospora. Comparison of the data on the whole breis on

a nitrogen basis show nearly four times as much activity on the average in the dormant spore extracts as in the extracts of the approximately 2-day-old (43- and 47-hour-old) mycelia. The average activity in the extracts from the approximately three-day-old (64-, 66-, 68- and 70-hour-old) mycelia is twice that of the 2-day-old mycelia but still far less than that of the dormant spore extracts. In general, good agreement for particular extracts is found between the two assay methods.

Fractionation on the mycelial brei showed, as expected, that nearly all (97 %) of the cytochrome oxidase activity was localized in the particles. As was true with the breis, the particles from the 3-day-old mycelia possessed more activity than their 2-day-old counterparts.

This apparent variation with age led to a study of the rate of growth of the mycelium of *N. tetrasperma*. The resulting growth curve is shown in figure 7. From examination of this curve it is apparent that the extracts prepared from the organism after 2-days' growth represent mycelium at the end of the log phase of growth while the 3-day-old mycelial cultures are well into the stationary phase. Therefore, the extracts of 2- and 3-day-old mycelia were of quite different physiological ages and variations in enzymatic activity are expected.

Discussion and Conclusions

Although the pyruvic oxidase enzyme system has been demonstrated in cell-free extracts of many higher plants, it was not possible to do so in extracts of Neurospora. In the fungi, Bonner and Machlis (3) were able to show the presence of this enzyme system in cell-free extracts of the mycelium of Allomyces and Nossal (22) and Linnane and Still (21) found it in yeast. Ramakrishnan (25) demonstrated activity in extracts of *Aspergillus niger* by following the reduction of 2, 6-dichlorophenol-indophenol, spectrophotometrically. The present work, however, is not the first failure to demonstrate this enzyme system as other unsuccessful attempts have been reported. Strauss (34) found that cell-free homogenates of mycelia of an acetate-requiring mutant of Neurospora did not oxidize pyruvate even when supplemented with cocarboxylase, DPN, ATP, and fumaric acid. However, he did obtain pyruvate oxidation with intact mycelia. Owens (24) was unable to obtain oxidation with cell-free extracts of Neurospora conidia although he supplemented the extracts with a number of different possible co-factors. On the other hand, oxygen uptake did occur when a dye, *p*-phenylenediamine (PPD), was present in the mixture. The addition of cytochrome c further stimulated oxygen uptake. This oxidation of pyruvate will be discussed later.

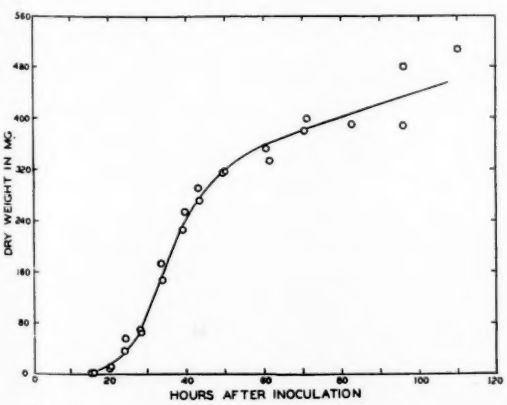


Fig. 7. Growth curve of Neurospora mycelium. Each circle represents the dry weight of mycelium obtained from one flask containing originally 65 ml of medium and 1 ml of conidial suspension.

Failure to extract an active pyruvate oxidase enzyme system from Neurospora can be explained in several ways. Loss of a necessary co-factor may have occurred although many known co-factors were added to the extract without stimulation of oxygen uptake. Inhibitory compounds may be released from the cell during preparation of the extract. Fatty acids in concentrations of 10^{-3} M and greater have been shown to inhibit pyruvate oxidation by rat kidney mitochondria (27). This is interesting in view of the fact that lipid appears to be the endogenous substrate for respiration during certain phases of the life cycle of Neurospora (20).

A TCA cycle acid, oxalacetate, in a concentration of 0.018 M, has been shown to temporarily inhibit the oxidation of pyruvate by avocado fruit particulates (1). At this concentration, oxalacetate inhibited oxidation of 0.02 M pyruvate for 80 minutes due, as the authors suggest, to a reaction in which the oxidation of pyruvate is coupled to the reduction of oxalacetate instead of oxygen.

That there is a strong pyruvic carboxylase present in these cell-free extracts is shown by this work and that of Sussman et al (36). Because both the pyruvic carboxylase and oxidase systems are derived from a common intermediate, the aldehyde diphosphothiamine enzyme complex (12), it appears that a competition may exist between these two enzyme systems in cell-free homogenates which may not reflect correctly their status in the intact cell. Sussman et al (36) detected no acetoin in cell-free extracts so this system is apparently not functioning here. Schwett et al (28) attempted to separate the oxidative from dismutative activity of the pyruvic oxidase enzyme system in pigeon breast muscle and in both the crude and most purified pyruvic oxidase systems, a constant ratio of non-oxidized to oxidized products was always maintained. Thus closely related enzymatic activities may interfere with measurements of the oxidative system.

Owens (24) was unable to obtain oxygen uptake in the presence of pyruvate with cell-free extracts of Neurospora conidia. However, when he added

p-phenylenediamine (PPD), a dye used for assaying cytochrome oxidase activity, oxygen uptake occurred. When he added cytochrome c with the PPD and pyruvate, oxygen uptake was even greater but with cytochrome c and PPD there was no oxygen uptake. The results reported here do not agree with this latter observation of Owens; we always obtained rapid oxygen uptake when PPD and cytochrome c were added to cell-free extracts. Inasmuch as this combination has been used in assays of cytochrome oxidase (31), the oxygen uptake with these addenda verifies the work of Sussman et al (36) who found this enzyme to be present in the ascospores. Owens (24) in the text of his paper indicated "the presence of active cytochrome oxidase" but the data in his table contradict this statement.

Owens suggested that the stimulation of pyruvate oxidation that is induced by PPD might not be due to the oxidation of pyruvate but of its decarboxylation product. The data present here are consistent with the suggestion that acetaldehyde is the decarboxylation product of pyruvate and that oxygen uptake in the presence of PPD and pyruvate or acetaldehyde is due, in part at least, to the oxidation of these compounds. Because PPD is oxidized in this system, it does not act analogously to ferricyanide or 2,6-dichlorophenolindophenol which may accept electrons from the aldehyde-enzyme complex to liberate the carbon skeleton as free acetic acid (12). Thus the unequivocal oxidation of pyruvate by cell-free extracts of Neurospora has yet to be demonstrated.

Evidence presented here indicates that oxygen uptake when PPD is added to mycelial extracts is due to the oxidation of reduced endogenous cytochrome c by cytochrome oxidase rather than to laccase. Cheng (5) had earlier reported the absence of laccase in mycelial extracts on the basis of slow oxidation of PPD. This slow oxidation is attributed here to the action of cytochrome oxidase on endogenous cytochrome c. This indirect evidence is backed up by the direct spectroscopic observation of cytochrome c in mycelial particles. This agrees with earlier observations of cytochrome c in *Neurospora crassa* (4, 39). Haskins et al (14) found that the whole mycelia of *N. crassa* contained 0.15 to 0.22 % cytochrome c on a dry weight basis. Unfortunately it is not possible to compare directly these results with those reported here. The very low rates of oxygen uptake when PPD is added to ascospore extracts suggests that there is a low level of endogenous cytochrome c in those extracts. A possible alternative explanation is that the apparent lack of cytochrome c in the extracts demonstrated by the indirect manometric method does not correctly represent the concentration of cytochrome c in the intact cell. Unfortunately, the direct spectroscopic examination of the particles from the spores does not give us any helpful information since although no cytochrome c was observed, it was not possible to obtain enough material for a comparable sample size to that obtained from mycelia.

The endogenous Qo_2 of *N. crassa* mycelium was found to be 29.6 (15) while that of dormant ascospore was 0.25 to 0.59 (11). Thus it would not be surprising to find more cytochrome c in the mycelia than in the ascospores if the cytochrome system is the principal oxidative system in this organism. The indirect manometric data is consistent with this suggestion. A parallel in insect tissue was reported by Shappirio and Williams (29), who found no detectable cytochrome c in the heart of dormant pupa of the Cercropia silkworm while in the late developing adult and mature adult moderate amounts were found.

The presence of an enzyme capable of oxidizing *p*-phenylenediamine, interpreted here to be cytochrome oxidase and endogenous cytochrome c, was detected in Neurospora by Tissieres and Mitchell (39). They found oxygen uptake in the presence of PPD alone ($Qo_2 = 25$), while with cytochrome c added the Qo_2 increased to 450. With ascorbate as the reducing agent in place of PPD, the values were 10 and 472, respectively. The fact that oxidation of ascorbate alone was less intense than that of PPD alone agrees with the statement of Slater that endogenous cytochrome c can be reduced rapidly by PPD but not by ascorbic acid.

The Qo_2 for cytochrome oxidase of 340 obtained in the present work with extracts of 4-day-old mycelium compares reasonably well with the 450 obtained by Tissieres and Mitchell (39) with extracts of 4- to 5-day-old mycelia of *N. crassa* at the higher temperature of 35° C. On the other hand, Cheng (5) reported 665 using hydroquinone as the reducer with extracts of younger (1.5-day-old) mycelium of *N. tetrasperma* at 25° C.

The fact that the V_{max} values for cytochrome oxidase are greater in the brei from dormant ascospores than in the mycelial brei suggests that in the former this enzyme is not the limiting factor in the electron transport system. Cytochrome c, in contrast, may be a limiting factor in the dormant ascospores and one consequence of the activation of ascospores may be an increase in the amount of cytochrome c.

Because the manometric and spectophotometric methods of assaying cytochrome oxidase activity are based on different principles, it was of interest to compare the two assay procedures on the same enzyme preparations. The data in table IV demonstrate that quite good agreement between the two procedures was obtained with extracts of tissues up to 3 days old.

The literature contains several reports of the comparison between the two assay methods (6, 7, 8). Except in the experiments of Fritz and Beevers (8), discrepancies were found between the two methods. Because that work and the results reported here were the only studies comparing methods in which different cytochrome c concentrations were used and a V_{max} value obtained, it is suggested that the lower values of cytochrome oxidase activity obtained with the manometric method in the past was due to lower than optimal cytochrome c concentrations.

On the other hand, Smith and Conrad (32) sug-

gested that a reason for disagreement between the two assay methods was that concentrations of cytochrome c often used in manometric assays inhibit cytochrome oxidase. They contended that the method of extrapolation to infinite concentration used by Slater actually measured a greatly inhibited cytochrome oxidase activity. The fact that results of Fritz and Beevers and those reported here show good agreement between the two methods suggests that at the concentrations of cytochrome c used in these studies no inhibition was occurring. The lack of agreement in the assays of these older mycelia could also be ascribed to the presence of an enzyme which was not present earlier and which was assayed in the spectrophotometric but not the manometric procedure.

Summary

Attempts to demonstrate the oxidation of pyruvate by cell-free extracts of ascospores and mycelium of *Neurospora tetrasperma* by any of the commonly used methods of extraction and mixtures of co-factors were unsuccessful. Adding a dye, *p*-phenylenediamine (PPD) did stimulate oxygen uptake when pyruvate was added to extracts of dormant and activated ascospores, conidia, and mycelia. Further experiments showed that this oxygen uptake is probably due not to oxidation of pyruvate but to oxidation of the decarboxylation product of pyruvate, acetaldehyde, formed from pyruvate by the carboxylase in these extracts. Acetaldehyde was oxidized by these extracts enzymatically but only in the presence of PPD.

Oxygen uptake by mycelial extracts in the presence of PPD alone has been shown to be due to the oxidation of endogenous cytochrome c by cytochrome oxidase. The endogenous cytochrome c content was measured spectroscopically. Lack of oxygen uptake by extracts of dormant ascospores in the presence of PPD suggests an absence of endogenous cytochrome c in these extracts. On the other hand, the level of cytochrome oxidase is higher in the dormant ascospore extracts than in the mycelial extracts. If the extracts represent accurately the status of these enzymes in the intact cells, it can be inferred that the level of cytochrome c and not the level of cytochrome oxidase may be one factor accounting for the low rate of respiration in the dormant ascospores.

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Literature Cited

1. Avron (Abramsky), M. and J. B. Biale. 1957. Metabolic processes in cytoplasmic particles of the avocado fruit. V. Effect of oxalacetate on the oxidation of pyruvate and succinate. Jour. Biol. Chem. 225: 699–708.
2. Ball, E. G. and Octavia Cooper. 1957. Observations on the function of cytochromes c and c_1. Jour. Biol. Chem. 226: 755–763.
3. Bonner, B. A. and L. Machlis 1957. Respiration of the mycelia and mitochondria of the filamentous watermold, *Allomyces macrogynus*. Plant Physiol. 32: 291–301.
4. Boulter, D. and E. Derbyshire. 1957. Cytochromes of fungi. Jour. Exp. Bot. 8: 313–318.
5. Cheng, S. C. 1954. The terminal respiratory enzymes in *Neurospora tetrasperma*. Plant Physiol. 29: 458–467.
6. Cooperstein, S. J. and A. Lazarow. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. Jour. Biol. Chem. 189: 665–670.
7. Frajola, W. J. and C. S. Vestling. 1954. A kinetic study of cytochrome oxidase. Jour. Biol. Chem. 209: 677–686.
8. Fritz, G. and H. Beevers. 1955. Cytochrome oxidase content and respiratory rates of etiolated wheat and barley seedlings. Plant Physiol. 30: 309–317.
9. Goddard, D. R. 1935. The reversible heat activation inducing germination and increased respiration in the ascospores of *Neurospora tetrasperma*. Jour. Gen. Physiol. 19: 45–60.
10. Goddard, D. R. 1939. The reversible heat activation of respiration in Neurospora. Cold Spring Harbor Symposia on Quant. Biol. 7: 362–376.
11. Goddard, D. R. and P. E. Smith. 1938. Respiratory block in the dormant spores of *Neurospora tetrasperma*. Plant Physiol. 13: 241–264.
12. Gunsalus, I. C. 1954. Group transfer and acyl-generating functions of lipoic acid derivatives. In: The Mechanism of Enzyme Action, W. D. McElroy and B. Glass, eds. Pp. 545–580. Johns Hopkins Press, Baltimore.
13. Hartree, E. F. 1955. Haematin compounds. In: Modern Methods of Plant Analysis, K. Paech and M. V. Tracey, eds. Vol. 4. Pp. 197–245. Springer-Verlag, Berlin.
14. Haskins, F. A., A. Tissieres, H. K. Mitchell, and Mary B. Mitchell. 1953. Cytochromes and the succinic acid oxidase system of poky strains of Neurospora. Jour. Biol. Chem. 200: 819–826.
15. Heplar, J. Q. and E. L. Tatum. 1954. Some factors affecting carbon dioxide metabolism in *Neurospora crassa*. Jour. Biol. Chem. 208: 489–494.
16. Holton, R. W. and A. S. Sussman. 1957. Pyruvate oxidation and electron transport in extracts of Neurospora. Plant Physiol. 32 suppl.: xxx.
17. Koch, F. C. and T. L. McMeekin. 1924. A new direct Nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. Jour. Amer. Chem. Soc. 46: 2066–2069.
18. Lieberman, M. and J. B. Biale. 1956. Co-factor requirements for oxidation of alpha-keto acids by sweet potato mitochondria. Plant Physiol. 31: 425–429.
19. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666.
20. Lingappa, B. T. and A. S. Sussman. 1959. Endogenous substrates of dormant, activated, and germi-

nating ascospores of *Neurospora tetrasperma*. Plant Physiol. 34: 466–472.
21. LINNANE, A. W. and J. L. STILL. 1955. The isolation of respiring mitochondria from baker's yeast. Arch. Biochem. Biophys. 59: 383–392.
22. NOSSAL, P. M. 1954. Oxidative reactions in cell-free yeast extracts. Biochim. Biophys. Acta 14: 154–155.
23. OWENS, R. G. 1955. Metabolism of fungus spores. I. Oxidation and accumulation of organic acids by conidia of *Neurospora sitophila*. Contrib. Boyce Thompson Inst. 18: 125–144.
24. OWENS, R. G. 1955. Metabolism of fungus spores. II. Cytochrome oxidase, succinoxidase, and pyruvate carboxylase systems in homogenates of conidia of *Neurospora sitophila*. Contrib. Boyce Thompson Inst. 18: 145–152.
25. RAMAKRISHNAN, C. V. 1954. The tricarboxylic acid cycle in *Aspergillus niger*. Enzymologia 17: 169–174.
26. RYAN, F. J. 1950. Selected methods of Neurospora genetics. In: Methods in Medical Research, R. W. Gerard, ed. Vol. 3. Pp. 51–75. Year Book Publishers, Inc., Chicago.
27. SCHOLEFIELD, P. G. 1956. Studies on fatty acid oxidation. IV. The effects of fatty-acids on the oxidation of other metabolites. Can. Jour. Biochem. Physiol. 34: 1211–1225.
28. SCHWEET, R. S., MARIA FULD, KATHERINE CHESLOCK, and M. H. PAUL. 1951. Initial stages of pyruvate oxidation. In: Phosphorous Metabolism, W. D. McElroy and B. Glass, eds. Vol. 1. Pp. 246–259. Johns Hopkins Press, Baltimore.
29. SHAPPIRIO, D. G. and C. M. WILLIAMS. 1957. The cytochrome system of the Cercropia silkworm. I. Spectroscopic studies of individual tissues. Proc. Royal Soc. (London) B 147: 218–232.
30. SHEAR, C. L. and B. O. DODGE. 1927. Life histories and heterothallism of the red bread mold fungi of the *Monilia sitophila* group. Jour. Agric. Research 34: 1019–1042.
31. SLATER, E. C. 1949. The measurement of the cytochrome oxidase activity of enzyme preparations. Biochem. Jour. 44: 305–318.
32. SMITH, LUCILE and HELEN CONRAD. 1956. A study of the kinetics of the oxidation of cytochrome c by cytochrome c oxidase. Arch. Biochem. Biophys. 63: 403–413.
33. STOTZ, E. 1943. A colorimetric determination of acetaldehyde in blood. Jour. Biol. Chem. 148: 585–591.
34. STRAUSS, B. S. 1952. Aspects of the carbohydrate metabolism of a mutant of *Neurospora crassa* requiring acetate for growth. Arch. Biochem. Biophys. 36: 33–47.
35. SUSSMAN, A. S. 1954. Changes in the permeability of ascospores of *Neurospora tetrasperma* during germination. Jour. Gen. Physiol. 38: 59–77.
36. SUSSMAN, A. S., J. R. DISTLER, and J. S. KRAKOW. 1956. Metabolic aspects of Neurospora activation and germination. Plant Physiol. 31: 126–135.
37. SUSSMAN, A., R. HOLTON, and BERTA VON BÖVENTER-HEIDENHAIN. 1958. Physiology of the cell surface of Neurospora ascospores. Entrance of anions and non-polar compounds. Arch. Mikrobiol. 29: 38–50.
38. SUSSMAN, A. S. and B. T. LINGAPPA. 1959. Role of trehalose in ascospores of *Neurospora tetrasperma*. Science 130: 1343.
39. TISSIERES, A. and H. K. MITCHELL. 1954. Cytochromes and respiratory activities in some slow growing strains of Neurospora. Jour. Biol. Chem. 208: 241–249.
40. WALKER, D. A. and H. BEEVERS. 1956. Some requirements for pyruvate oxidation by plant mitochondrial preparations. Biochem. Jour. 62: 120–127.

FACTORS INFLUENCING IN VIVO FORMATION OF *p*-HYDROXYMANDELONITRILE-β-D-GLUCOSIDE [1, 2, 3]

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The aglycone portions of several of the naturally occurring cyanogenic glucosides are known to be aromatic and to contain a nitrile group. Little is known concerning the biosynthesis of either the aglycone or its nitrile moiety, or of the reaction in which glucose is transferred to the aglycone or its derivative. The glucoside, *p*-hydroxymandelonitrile-β-D-glucoside (*p*-HMN-β-G), was first isolated from *Sorghum vulgare* Pers. and characterized by Dustan and Henry in 1902 (7). The glucoside may be hydrolyzed by either weak acid or emulsin, an enzyme preparation containing a β-glucosidase and an oxynitrilase which cleaves the glucose from the aglycone and the nitrile moiety from *p*-hydroxymandelonitrile (*p*-HMN) (1). The products of the emulsin-catalyzed *p*-HMN-β-G hydrolysis are 1 mole each of glucose, HCN, and *p*-hydroxybenzaldehyde.

Rosenthaler in 1923 suggested L-tyrosine as a precursor of *p*-HMN-β-G based upon his observation that plants fed tyrosine had a greater cyanide content than controls not fed tyrosine (13). More recently, Swanson and Shaw (15) suggested that nitrate is converted to the nitrile nitrogen, since they observed that 2, 4-dichlorophenoxyacetic acid (2, 4-D) inhibits the formation of the cyanogenic glucoside when sprayed on sudan grass and that the nitrate level of the plants increases.

Preliminary investigations in Conn's laboratory (6) and in this laboratory (8) have also indicated tyrosine to be a likely precursor of the aglycone portion of the glucoside.

This paper describes the chromatographic separation of the cyanogenic glucoside from other aromatic compounds in *Sorghum vulgare* variety Rox orange, and the influence of various aromatic and nitrogenous compounds upon the rate of formation of *p*-HMN-β-G in vivo.

MATERIALS

N-formyl glutamic acid and *N*-formyl aspartic acid were synthesized according to the procedure of Tabor and Mehler (16), followed by recrystallization twice from ethanol and benzene. The compounds were characterized by chromatography in tertiary butanol : formic acid : water (70 : 15 : 15), and identified by spraying the chromatogram with a 1 per cent starch, 1 per cent KI solution (14).

p-Hydroxyphenylserine[5] was prepared according to the procedure of Rosenmund and Dornsaft (12) giving a compound melting in the range 215 to 220° C.

p-Hydroxybenzaldehyde, mp 116 to 117° C, was purchased from Eastman Kodak Co., and recrystallized from water.

The amino acids, 2, 4-dichlorophenoxyacetic acid, and emulsin were purchased from Nutritional Biochemical Corp. All other chemicals and reagents used were of cp grade.

METHODS

CONDITIONS FOR GROWING CYANIDE-LOW SORGHUM: Preliminary investigations indicated that sorghum germinated under atmospheric conditions in either the light or dark contained relatively high concentrations of cyanide, present as a cyanogenic glucoside. Conditions were sought which would allow germination of the seed without formation of *p*-HMN-β-G in seedlings. The coleoptiles from seeds allowed to germinate in the dark in an atmosphere of water pumped nitrogen, (0.2 % O_2), contained less than 1 μg of cyanide per 100 mg fresh weight after 5 days under these conditions. Seedlings germinated in air normally contain about 40-fold more cyanide.

EXPERIMENTAL CONDITIONS: All experiments in which *p*-HMN-β-G formation was estimated, based upon the HCN released by emulsin, were carried out with tissue obtained from 5-day-old (cyanide-low) sorghum seedlings. This tissue was obtained by carefully severing the shoot from the seed and roots and transferring the coleoptiles to Petri dishes containing 20 ml of a sterile non-nitrogenous nutrient solution. The nutrient solution contained $CaCl_2$, 2×10^{-3} M; KH_2PO_4, 1×10^{-3} M; $MgSO_4$, 1×10^{-3} M; $FeSO_4 \cdot 7H_2O$, 2 mg/liter; $ZnSO_4 \cdot 7H_2O$, 1 mg/liter; and H_3BO_3, 1 mg/liter. All experiments were conducted in the light at 27° C unless otherwise stated.

Sorghum seedlings 5 days old and germinated in the dark under atmospheric conditions were used in the chromatographic separation of the glucoside from other plant constituents.

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[5] *p*-Hydroxyphenylserine was prepared by Mr. R. D. Gaines.

CYANIDE DETERMINATION: The tissue was macerated in two volumes of water, heated at 100° C for 5 minutes, the solution adjusted to pH 6, and incubated with 10 mg of emulsin for 4 hours at 30° C in a stoppered test tube. The reaction mixture was then adjusted to pH 10 with NaOH, filtered, and the NaCN in solution estimated by the method of Aldridge (2) as modified by Hudson and Pollack (10). This consists of converting the cyanide to cyanogen bromide followed by reacting the cyanogen bromide with benzidine in acidic pyridine, and measuring the colored complex which absorbs maximally at 520 mμ. This method will detect 0.3 μg of cyanide. The color intensity follows Beer's law from 0.5 to 5.0 μg.

RESULTS

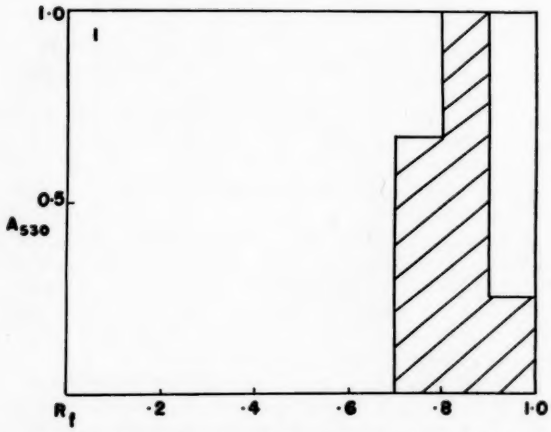
An alcoholic extract of 5-day-old etiolated sorghum tissue was chromatographed in butanol: pyridine: water (6:4:3) by allowing the solvent to ascend the paper. Figure 1 shows the portion of the chromatogram containing material which releases HCN upon treatment with emulsin. Table I shows the R_f's of various materials when strips from the same chromatogram were treated with ammoniacal silver nitrate, ninhydrin, 2, 4-dinitrophenylhydrazine in HCl, 2, 4-dinitrophenylhydrazine after treatment with emulsin or diazotized sulfanilic acid, represented by spray reagents A, B, C, C¹, and D, respectively. The table shows that sorghum contains no amino acids which migrate as rapidly as the glycoside. The glycoside is known to be easily hydrolyzed by alkali (4), and presumably the reducing material R_f 0.85 observed after spraying the chromatogram with ammoniacal silver nitrate results from the alkaline cleavage of the glycoside giving both *p*-hydroxybenzaldehyde and glucose. The strip of the chromatogram sprayed with 2, 4-dinitrophenylhydrazine showed a spot which migrated with an R_f of 0.9

TABLE I

R_f's OF SUGARS, AMINO ACIDS, ALDEHYDES, AND KETONES, AND HYDROXYLATED AROMATIC COMPOUNDS FOUND IN ALCOHOLIC EXTRACT OF SORGHUM

AROMATIC COMPOUNDS	SPRAY REAGENT*				
	A	B	C	C¹	D
	R_f	R_f	R_f	R_f	R_f
	0.08	0–0.01			0.10
		0.13			
		0.15			
	0.20	0.18			
		0.35			
Tyrosine	0.40				0.40
		0.45			
		0.55			
p-HMN-β-G	0.85				
p-OH Benzaldehyde			0.90	0.80–0.90	0.80–0.90

* Spray reagents: A, 0.5 M $AgNO_3$ in 3 *N* NH_4OH; B, 0.25 % ninhydrin in acetone (w/v); C, 0.5 saturated solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl; C¹, 10 mg/ml of emulsin in H_2O followed by incubation for 30 minutes at 30° C in an atmosphere saturated with H_2O, followed by drying and spraying with reagent C; D, diazotized sulfanilic acid in Na_2CO_3 (3).



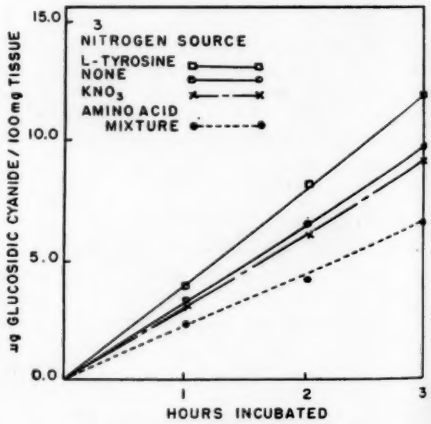


FIG. 1. Shows the area of the chromatogram which contained cyanide released by emulsin after an extract of etiolated sorghum was chromatographed in butanol: pyridine: H_2O (6:4:3). A portion of the chromatogram was cut into ten strips, each strip was eluted with H_2O and the cyanide in the eluate released by the action of emulsin. The cyanide was estimated by the procedure of Aldridge (2).

FIG. 2. Glucosidic cyanide yielded per 100 mg of tissue incubated in the dark or the light at 27° C for various intervals. No cyanide could be detected unless the heated sorghum extract was first incubated with emulsin. Cyanide-low sorghum seedlings described in the text were used.

which coincided with the rate of migration of *p*-hydroxybenzaldehyde in this solvent. If the strip was first sprayed with emulsin, then incubated 30 minutes at 30° C in an atmosphere saturated with H_2O and then sprayed with 2, 4-dinitrophenylhydrazine, the glucoside area contained material which reacted with 2, 4-dinitrophenylhydrazine. This indicates that treatment with emulsin released aldehydes or ketones. The strip of the chromatogram sprayed with diazotized sulfanilic acid (3) shows that etiolated sorghum contains relatively large quantities of two aromatic compounds, one migrating with an R_f 0.1 and the other 0.85. Tyrosine, R_f 0.4, was present in low concentrations. The reactivity of material migrating at R_f 0.85, toward diazotized sulfanilic acid is taken as further evidence that the cyanogenic glucoside is an aromatic hydroxylated compound.

Figure 2 shows the results of an experiment in which cyanide-low sorghum tissue was transferred to the nutrient solution and incubated in either the light or the dark at 27° C. The concentration of cyanide released by emulsin was determined after 24, 48, and 72 hours. The tissue exposed to light synthesized cyanogenic glucoside at a linear rate for 48 hours. Tissue incubated in the dark showed an increase in cyanogenic glucoside concentration for the first 24 hours but little increase in the next 48 hours. The rate of cyanogenic glucoside formation was not nearly as rapid in seedlings incubated in the dark as those incubated in the light. These data suggest that even though cyanogenic glucoside formation is not completely dependent upon photosynthesis, the rate of formation of the glucoside in a photosynthesizing plant is greater than in one not photosynthesizing.

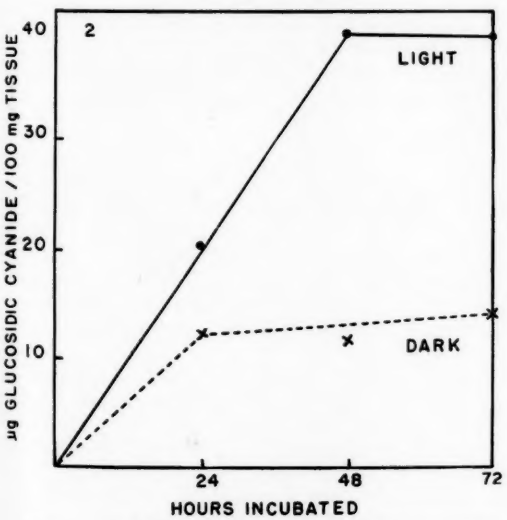


FIG. 3. The influence of various nitrogen compounds upon the quantity of glucosidic cyanide found after incubating cyanide-low sorghum for 1, 2, or 3 hours at 27° C in the light.

TABLE II

INFLUENCE OF VARIOUS COMPOUNDS UPON CYANOGENIC GLYCOSIDE FORMATION

COMPOUND*	μG HCN/100 MG TISSUE
None	41
p-Hydroxyphenylserine	74
p-Hydroxybenzaldehyde	60
L-Tyrosine	54
L-Serine	51
Tyramine	44
N-Formyl-L-aspartic acid	43
N-Formyl-L-glutamic acid	40
L-Histidine	36
L-Phenylalanine	33
L-Aspartic or L-Glutamic acids	30
L-Arginine	29

* All compounds were tested at a concentration of 1×10^{-3} M.

Figure 3 shows the results of an experiment in which KNO_3, a mixture of ten amino acids or L-tyrosine was added to the nutrient solution at a concentration of 1×10^{-3} M. Each of the amino acids (L-methionine, L-aspartic acid, L-glutamic acid, glycine, L-serine, L-phenylalanine, L-threonine, L-isoleucine, L-valine, and L-lysine) was present in a concentration of 1×10^{-4} M. This experiment shows that the rate of formation of the cyanogenic glucoside was approximately linear for the first 3 hours. The rate of glucoside formation was the greatest in the presence of L-tyrosine while the amino acid mixture caused a 25 per cent inhibition in the rate of glucoside formation compared to a control receiving no nitrogen compounds. Adding KNO_3 to the nutrient solution had no effect upon the rate of cyanogenic glucoside formation.

Further evidence that certain amino acids and aromatic compounds may influence glucoside formation is shown in table II. This table shows the concentration of glucosidic cyanide 48 hours after the seedlings were placed in a nutrient solution containing the compounds indicated. L-Aspartic acid, L-glutamic acid, L-arginine, L-phenylalanine, or L-histidine inhibited glucoside formation. The marked increase in cyanogenic glucoside formed when *p*-hydroxyphenylserine or *p*-hydroxybenzaldehyde was present in the nutrient solution suggests these compounds as intermediates in the biosynthesis of *p*-HMN-β-G. It is possible, however, that *p*-hydroxyphenylserine may in some manner interfere with the incorporation of tyrosine into protein which would make it appear as if *p*-hydroxyphenylserine was a precursor of the glycoside. The 50 per cent increase in glucoside formation in the presence of *p*-hydroxybenzaldehyde is difficult to understand unless the aldehyde is condensing with glycine or with cyanide. It is equally difficult to understand how L-serine could increase

TABLE III

INFLUENCE OF METABOLIC INHIBITORS UPON CYANOGENIC GLYCOSIDE FORMATION

INHIBITOR*	% INHIBITION OF CYANIDE FORMATION
Sodium azide	95
a, a′-Dipyridyl	90
Methylene blue	19
2,4-Dinitrophenol	14

* All inhibitors were present in the nutrient solution in a concentration of 1×10^{-3} M.

the formation of the glucoside, while a similar response when the plants were incubated in L-tyrosine is reasonable when the structure of the glucoside and L-tyrosine are compared.

Table III shows the results of experiments in which possible inhibitors were added to the nutrient solution on which sorghum tissue was floated and the cyanogenic glucoside content of the tissues measured after 24 hours. The presence of either methylene blue or 2, 4-dinitrophenol (2, 4-DNP) caused less than 20 per cent inhibition of cyanogenic glucoside formation. As the plant material used in these studies was intact tissue, the possibility that the potential inhibitors were not transported into the cells must be considered. It was observed, however, that the tissue-macerate from the 2, 4-DNP and methylene blue treated plants was deep yellow and dark blue respectively, indicating some of the material did get into the plants. The marked inhibition by sodium azide and a, a′-dipyridyl suggests enzymes containing iron are involved in the biosynthesis of the cyanogenic glucoside. Udenfriend and Cooper have shown that both azide and a, a′-dipyridyl inhibit the DPNH dependent enzymatic hydroxylation of phenylalanine to form L-tyrosine (17). The azide and dipyridyl inhibition of glucoside formation suggests L-tyrosine or some related compound becomes hydroxylated during the biosynthesis of the glucoside. Both sodium azide and a, a′-dipyridyl are also known to inhibit other types of reactions, and the observed inhibition of glucoside formation may be a secondary effect of the inhibitors.

DISCUSSION

The evidence presented in table II demonstrates that L-tyrosine, *p*-hydroxyphenylserine, serine, and *p*-hydroxybenzaldehyde permit an increase in the rate of formation of *p*-HMN-*β*-G, implicating these compounds as possible precursors in the biosynthesis of the glucoside. Although these experiments do not constitute direct evidence that these compounds are precursors of the glucoside, the structural relationships between each of them and the aglycone portion of the glucoside, coupled with the observed effect, make this postulate tempting. Recent information (unpubl. data) based upon the lack of incorporation of C^{14} from glycine-2-C^{14} into the nitrile carbon of the glucoside, indicates sorghum does not catalyze an aldol condensation of *p*-hydroxybenzaldehyde and glycine similar to the formation of phenylserine recently shown to occur in mammalian liver (5). Conn has recently shown (pers. communic.) that C^{14} from *p*-hydroxybenzaldehyde-C^{14} is incorporated into the aglycone portion of the glucoside. This finding, coupled with the observation that C^{14} from DL-tyrosine-2-C^{14} (8) is incorporated into the nitrile carbon of the glucoside, makes it appear the glycoside may be synthesized via two metabolic pathways. Further experiments are necessary to evaluate the relative importance of L-tyrosine and *p*-hydroxybenzaldehyde in the biosynthesis of *p*-HMN-*β*-G. The inhibition of glucoside formation by a mixture of the ten amino acids can be interpreted as further evidence for the role of L-tyrosine in the biogenesis of *p*-HMN-*β*-G. It is suggested that adding amino acids to the nutrient medium caused an increase in the rate of protein synthesis, and that this increased protein synthesis competed with glucoside biosynthesis for the limited amount of available intracellular L-tyrosine.

Although these data strongly suggest a role for *p*-hydroxyphenylserine in the biogenesis of the glucoside, it is conceivable that this compound acts indirectly. If *p*-hydroxyphenylserine acts as an effective inhibitor of L-tyrosine incorporation into plant protein, this would make more L-tyrosine available for glucoside formation.

The lack of effect of tyramine upon the amount of cyanogenic glucoside formed suggests that tyrosine is not decarboxylated, but rather reacts in some different manner, possibly hydroxylated to form *p*-hydroxyphenylserine, similar to the synthesis of norepinephrine from 3, 4-dihydroxyphenylethylamine (9, 11). The inhibition in gucoside formation by a, a′-dipyridyl or sodium azide also suggests an iron containing enzyme may be important in glucoside biosynthesis.

SUMMARY

I. The cyanogenic glucoside, *p*-HMN-*β*-G, present in sorghum was separated from the amino acids and free sugars by paper chromatography. Only one cyanogenic glucoside was found which would release HCN when incubated with emulsin. This compound migrated with an R_f of 0.8 in butanol : pyridine : water (6 : 4 : 3).

II. Conditions were described for germinating and growing Sorghum vulgare Pers. variety Rox Orange which gives seedlings containing 1/40th the concentration of cyanogenic glucoside found in seedlings allowed to germinate and grow in air.

III. Cyanogenic glucoside was synthesized more rapidly by plants exposed to light than by those which were kept in the dark.

IV. *p*-Hydroxyphenylserine, *p*-hydroxybenzaldehyde, L-tyrosine and L-serine increased the quantity of cyanogenic glucoside formed compared to a con-

trol which received a nitrogen free nutrient solution containing only inorganic salts. A mixture of ten amino acids or L-glutamic acid, L-aspartic acid, L-arginine, L-phenylalanine, or L-histidine alone inhibited the formation of the glucoside compared to the control.

V. a, a′-Dipyridyl and sodium azide caused a marked inhibition in the formation of *p*-HMN-*β*-G, while the presence of methylene blue or 2, 4-dinitrophenol in the nutrient solution caused only a slight inhibition in glucoside formed.

VI. The data are discussed in terms of the possibility that L-tyrosine and related compounds behave as precursors of the aglycone portion of the glucoside, with a, a′dipyridyl or sodium azide inhibiting a side chain hydroxylation.

Literature Cited

1. Albers, H., E. Baumann, and K. Myerbach. 1941. Oxynitrilase. Die Methoden der Fermentforschung 3: 2137–2149.
2. Aldridge, W. N. 1944. A new method for the estimation of micro quantities of cyanide and thiocyanate. Analyst 69: 262–265.
3. Armstrong, M. D., K. F. Shaw, and P. E. Wall. 1956. The phenolic acids of urine. Paper chromatography of phenolic acids. Jour. Biol. Chem. 218: 293–303.
4. Ballou, C. E. 1954. Alkali-sensitive glycosides. Advances in Carbohydrate Chem. 9: 59–95.
5. Bruns, F. H. and L. Fiedler. 1958. Enzymatische Spaltung und Synthese von L-threo-*β*-phenylserin and L-erythro-*β*-phenylserin. Biochem. Zeits. 330: 324–341.
6. Conn, E. E. and T. Akazawa. 1958. Biosynthesis of *p*-hydroxybenzaldehyde. Fed. Proc. 17: 205.
7. Dustan, W. R. and T. A. Henry. 1902. Cyanogenesis in plants, II. The great millet, *Sorghum vulgare*. Chem. News 85: 301.
8. Gander, J. E. 1958. In vivo biosynthesis of glycosidic cyanide in sorghum. Fed. Proc. 17: 226.
9. Hagen, P. J. 1956. Biosynthesis of norepinephrine from 3, 4-dihydroxyphenylethylamine (dopamine). Pharmacol. Exp. Therap. 116: 26.
10. Hudson, J. R. and J. R. A. Pollock. 1957. Determination of thiocyanate or cyanide in the presence of glycine. Analyst 82: 374.
11. Rosenfeld, G., L. C. Leeper, and S. Udenfriend. 1958. Biosynthesis of norepinephrine and epinephrine by the isolated perfused calf adrenal. Arch. Biochem. Biophys. 74: 252–265.
12. Rosenmund, K. W. and H. Dornsaft. 1919. Uber oxy- und Dioxphenylserin und die Muttersubstanz des Adrenalins. Ber. deut. Chem. Ges. 52: 1734–1749.
13. Rosenthaler, L. 1923. Zur Prufung der Treuleschen Hypothese. Biochem. Zeits. 134: 215–224.
14. Rydon, H. N. and P. W. G. Smith. 1952. A new method for the detection of peptides and similar compounds on paper chromatograms. Nature 169: 922–923.
15. Swanson, C. R. and W. C. Shaw. 1954. The effect of 2, 4-dichlorophenoxy-acetic acid on the hydrocyanic acid and nitrate content of sudan grass. Agron. Jour. 46: 418–421.
16. Tabor, H. and A. H. Mehler. 1954. Isolation of *N*-formyl-L-glutamic acid as an intermediate in the enzymatic degradation of L-histidine. Jour. Biol. Chem. 210: 559–568.
17. Udenfriend, S. and J. R. Cooper. 1952. The enzymatic conversion of phenylalanine to tyrosine. Jour. Biol. Chem. 194: 503–511.